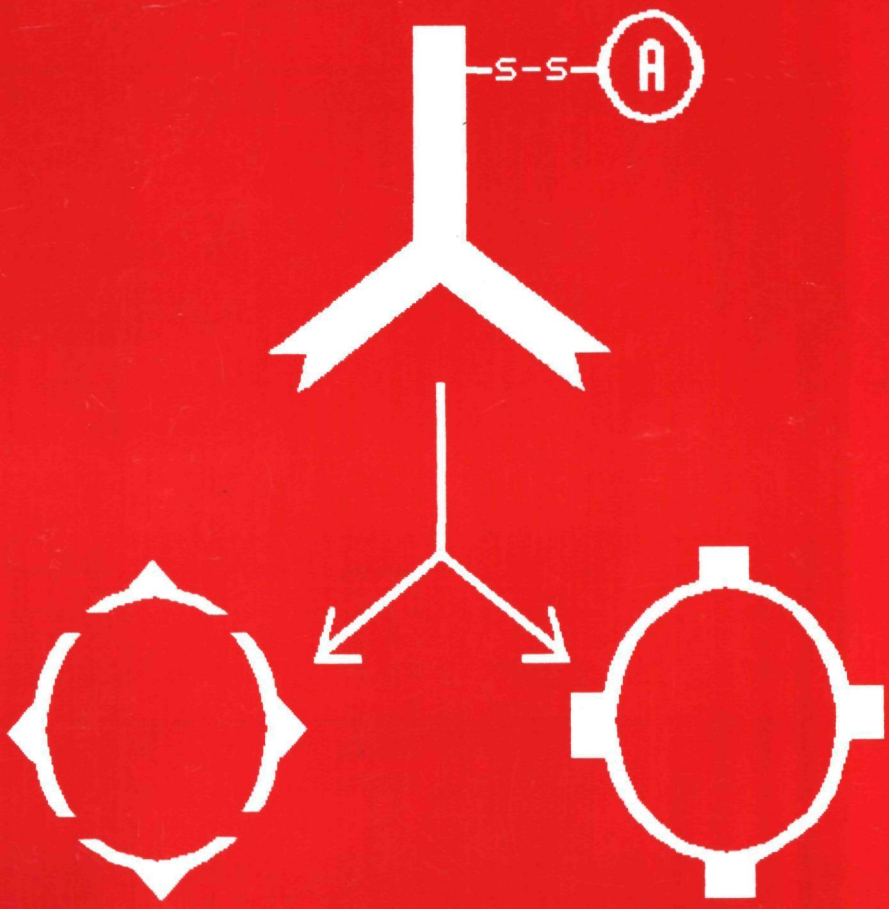
EX VIVO ELIMINATION OF NORMAL AND MALIGNANT T CELLS FROM BONE MARROW GRAFTS BY RICIN A-CHAIN IMMUNOTOXINS



F.W.M.B. Preijers

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Een wetenschappelijke proeve op het gebied van de geneeskunde en tandheelkunde

PROEFSCHRIFT

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PROF DR C HAANEN
PROF DR P J A CAPEL (RU Utrecht)

CO-REFERENTEN:

DR T DE WITTE
DR. J M C WESSELS

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Aan Lucie en Tim,

Ter herinnering aan
mijn vader.

CONTENTS:

ABBREVIATIONS:

BFU-E	burst forming unit of erythrocytes
BMT	bone marrow transplantation
CD	cluster of differentiation
CFU-GEMM	colony forming unit of granulocytes, erythrocytes, megakaryocytes, and monocytes
CFU-GM	colony forming unit of granulocytes and monocytes
Con A	concanavalin A
CR	complete remission
ELISA	enzyme-linked immunosorbent assay
FCM	flow cytometry
FCS	fetal calf serum
GVHD	graft-versus-host disease
ID50	dose required for 50% inhibition of protein synthesis
IL-2	interleukin-2
IT	immunotoxin
MoAb	monoclonal antibody
PHA	phytohaemagglutinin
SDS	sodium dodecyl sulphate
SPDP	N-succinimidyl 3-(2-pyridyldithio)propionate
T-ALL	acute T cell lymphoblastic leukemia
T-LL	T cell lymphoblastic lymphoma

CHAPTER 1

INTRODUCTION

Recent advances in understanding the biology and therapy of leukemia and lymphoma have a considerable impact on the survival in this heterogeneous group of diseases. Long-term disease free survival rates (>3 years) of 10-60% for patients with acute leukemia depending on age are now attained in patients who receive chemotherapy [1]. Nevertheless, many relapses occur and in adults the survival beyond 5 years is still low [2]. Most patients who fail to respond to primary chemotherapy have a very poor prognosis with this conventional therapy [3,4]. The anti-leukemic effect of chemotherapy and radiation is related to the dose which is limited by concomitant myelosuppression. The property of bone marrow cells to home in the bone marrow compartment allows a repopulation and restoration of the hematopoiesis after the intravenous infusion of bone marrow cells. Infusion of donor or autologous bone marrow cells makes feasible treatment with supralethal doses of chemo-radiotherapy and to rescue the patient with bone marrow transplantation (BMT). Presently BMT offers the best chance of long-term disease free survival in acute leukemias and highly malignant lymphomas [1,5].

Allogeneic bone marrow transplantation

The first bone marrow tranplantation was performed in animals by Lorenz and colleagues [6]. They discovered that supralethally irradiated mice could be fully reconstituted hematologically and immunologically by intravenous infusion of syngeneic bone marrow. Investigators in the late fifties demonstrated that allogeneic bone marrow transplantation could also be successful if the recipient strains of mice had been matched for the major histocompatibility complex (MHC) antigens [7,8]. After the identification of the human MHC (HLA) in 1968, the first successful BMT was performed with a HLA-identical sibling donor bone marrow [9]. Since 1970 BMT is increasingly used to treat severe immune deficiencies, aplastic anemia and leukemias. At present, world wide more than 22,000 patients have been treated with allogeneic BMT [10]. However, opportunistic infections, interstitial pneumonitis, graft-versus-host disease (GVHD), graft rejection and leukemic relapse remain obstacles to the success of BMT. GVHD is one of the major problems. Moderate to severe acute GVHD occurs in 30-70% of HLA-identical recipients with a

fatal outcome in approximately 50% of the severely affected patients despite posttransplant treatment with immunosuppressive drugs such as cyclosporin A and methotrexate [11,12,13]. GVHD is primarily mediated by immunocompetent lymphocytes present in the graft [14,15]. Ex vivo elimination of T cells from the marrow graft improves the clinical outcome of BMT by causing a reduction of the incidence and the severity of GVHD [16-18]. In this regard, T cell depletion has been a major advance. Unfortunately, elimination of high numbers of T cells results in a delayed bone marrow reconstitution, or even failure of engraftment or late graft rejection. Graft failure, largely due to immunologic rejection, increases from <1% with unmodified bone marrow grafts to approximately 15% with grafts of T cell depleted bone marrow [17,19,20]. In those cases where bone marrow grafting was used for the treatment of leukemia, the incidence of relapse appears significantly increased after transplantation of a T cell depleted graft [17]. Data from experiments in animals suggest that allogeneic BMT confers a graft-versus-leukemia (GVL) effect mediated by T cells that prevent leukemia relapse [21,22]. Though, it is unknown whether a similar phenomenon applies to man, data of relapse rates of 60% in identical twin BMT (syngeneic BMT), in which in contrast to allogeneic BMT no differences exist in the minor histocompatibility antigens, suggest the existence of GVL effect in man [23]. If it would be possible to eliminate T cell subpopulations responsible for the GVHD in the bone marrow, without simultaneous depletion of those cells responsible for GVL effect, an improvement of the clinical outcome of allogeneic BMT may be expected.

More than 70% of the patients who might benefit from a BMT, lack a HLA-compatible sibling donor. Transplant-related complications such as GVHD, graft rejection and infections due to the postgraft immunodeficiency, increase the mortality after BMT and limit the applicability to younger patients (<45 years). Therefore, allogeneic BMT remains restricted to approximately 15% of patients with acute leukemia and even a lower percentage of patients with chronic leukemia or lymphoma [24].

Autologous bone marrow transplantation

Autologous BMT (ABMT), in which the patient acts as his own marrow donor, may circumvent several drawbacks of allogeneic BMT. GVHD and its

related complications do not occur and the immunodeficiency is less profound, but malignant cells may be present in the graft and the GVL effect is absent [25]. Nevertheless the clinical application of ABMT increases steadily [26]. Though ABMT has obvious advantages for patients in whom conventional therapy has failed [27,28], relapse rate is higher than in allogeneic BMT [25,29]. This is partly due to the absence of GVHD and GVL effect as was concluded in a study comparing data of allogeneic and syngeneic BMT [23]. Moreover, the contaminating leukemic cells in the reinfused autologous bone marrow may contribute to the increased incidence of relapse. The ability to "purge" marrow from malignant cells may decrease the number of relapses after ABMT.

2. APPROACHES FOR THE ELIMINATION OF NORMAL OR MALIGNANT T CELLS FROM BONE MARROW

In the last few years many methods have been developed to eliminate T cells from bone marrow. Methods that result in a partial depletion of T cells, such as counterflow centrifugation [18] or rosetting with sheep erythrocytes followed by agglutination with soybean agglutinin [30], have been applied successfully in allogeneic BMT. However, only those methods which achieve a specific and complete elimination of malignant T cells are suitable for purging of autologous bone marrow and can be used for selective elimination of T cell subpopulations from donor bone marrow. Cytostatic agents such as Asta Z or 4-hydroperoxycyclophosphamide have been used for this purpose, albeit with substantial loss of hematopoietic progenitor cells [31,32]. With the development of hybridoma technology by Kohler and Milstein [33] it became possible to produce specific monoclonal antibodies (MoAbs) directed against differentiation antigens or specific tumor antigens expressed on normal or malignant cells. MoAbs as such are not toxic to cells. In combination with complement, however, cells can be killed effectively and an elimination of 3- to 4-logs can be achieved [34-36]. Although the use of MoAb with complement is an attractive concept, a number of problems can arise when applied to human bone marrow. Firstly, not all MoAbs fix complement, and more importantly most MoAbs do not fix human complement which necessitates the use of nonspecific xenogeneic complement sources

such as rabbit serum. Secondly, complement activity present in rabbit sera displays great batch variations. Thirdly, high cell number, erythrocytes and unsubstituted cell surface sialic acid residues can activate complement resulting in an undesired complement consumption and a decreased complement-mediated killing [37]. Fourthly, lysed cells cause clumping and are difficult to remove from the cell suspension. Finally, fragments of the complement cascade are profound immunoregulators with unknown effects on hematopoiesis.

Approximately 80 years ago Paul Ehrlich put forward the original idea of the "magic bullet", a molecule consisting of a toxic compound attached to an antibody. This approach may circumvent the problems with complement. Many drugs [38,39,62] and radioisotopes [40] have been conjugated with MoAbs to increase their efficacy. High potency, however, will only be beneficial in tumor therapy if it is specific for the target cell, in the sense that the effector function remains inactive outside the cell and becomes activated only after binding to and entering the target cell. Over the last few years such molecules, named immunotoxins (ITs), have been constructed by conjugating toxins from bacteria or plants to MoAbs. Many investigators have contributed insights into the mechanisms by which these toxins kill cells [41,42]. The toxins most widely utilized are diphteria toxin, the exotoxin secreted by Corynebacterium diphteria, and the plant toxin ricin from the seeds of Ricinus communis. Besides, abrin, gelonin pokeweed antiviral protein (PAP), saporin or pseudomonas exotoxin are used as toxic subunit of the IT. Toxins, such as ricin, consist of two chains: the toxic A-chain and the binding sites containing B-chain. The B-chain is a galactose-specific lectin which binds to virtually all eukaryotic cells followed by endocytotosis. This facilitates the penetration of the A-chain into the cytoplasm [43]. The A-chain is a N-glycosidase that cleaves the N-glycosidic bond of a particular adenoside residue from ribosomal RNA and inhibits strongly and irreversibly the protein synthesis of the target cell [44]. No more than 1800 ricin molecules bound to the cell surface seem to be required for the penetration into the cytoplasm of at least one A-chain which is sufficient to kill the cell [45]. Immunotoxins prepared with intact toxins are extremely potent cytotoxic agents in vitro for cells carrying the appropriate target antigen, often matching or even surpassing the toxicity of the native

toxin [46]. However, though the nonspecific binding by the B-chain can be blocked in vitro by high concentrations of sugars such as galactose and lactose [47,48], these sugars are rapidly excreted in vivo [49] and the release of whole ricin from conjugates after infusion of treated marrow may be hazardous to the patient [50]. High concentrations of galactose or lactose are toxic for animals so that blocking of binding sites does not appear to be feasible in human beings. Therefore, the galactose binding sites have to be blocked by other agents than sugars [49] or the A-chain has to be separated from the B-chain and coupled to a MoAb, which replaces the function of the B-chain by enhancing the internalization of the A-chain. The low toxicity of ricin A-chain conjugates in vivo (LD50 in mice: 22.3 mg/kg) compares favourably to intact ricin conjugates (LD50 in mice: 0.016 mg/kg) [51].

The ricin A-chain ITs kill more slowly than ITs with intact ricin, which makes it desirable to develop methods to increase their potency. Electron microscopic investigations suggested that the IT might be destroyed in the secundary lysosomes [52,53]. Therefore the influence of lysosomotrophic agents was studied. These investigations showed that ammonium chloride and carboxylic ionophores have a highly potentiating effect on the cytotoxicity of ricin A-chain ITs. This resulted in an efficacy even exceeding that of intact ricin [54]. Moreover, the choice of the MoAb will influence the cytotoxic potential of the IT [63,64].

3. PRODUCTION OF RICIN A-CHAIN IMMUNOTOXINS

The A-chain (Mw 30,000) and the B-chain (Mw 33,000) of ricin (Mw 63,000) can be dissociated by reduction of the disulfide bridge between the two ricin chains with β-mercaptoethanol [55]. The A-chain can be purified by chromatography on DEAE-cellulose. Contaminating B-chains can be removed by lectin affinity chromatography or by antibody affinity chromatography (Sepharose-coupled antibody to the B-chain in the presence of lactose) [55]. If ricin is dissociated in the two subunits it shows 100-times higher cytotoxicity in a cell free system of isolated ribosomes as compared to the intact form, suggesting that in the intact ricin molecule the A-chain is sterically hindered by the B-chain [56].

The intersubunit disulfide bridge appears to be essential for the cytotoxicity of the ricin and plays a role in the transmembrane transport of the ricin A-chain [57], implicating that the ricin A-chain has to be conjugated to a MoAb by a disulfide linkage. The importance of the nature of the linkage was supported by the finding that a more stable thioether cross-link decreased the cytotoxicity of the IT [48,58]. However, by the lack of a free sulfide group in the MoAb an artificial disulfide bridge has to be created by a heterobifunctional cross-linker. N-succinimidyl 3-(2 pyridyldithio) propionate (SPDP) is commonly used for this purpose [59]. The preparation of ITs with SPDP is described in figure 1.

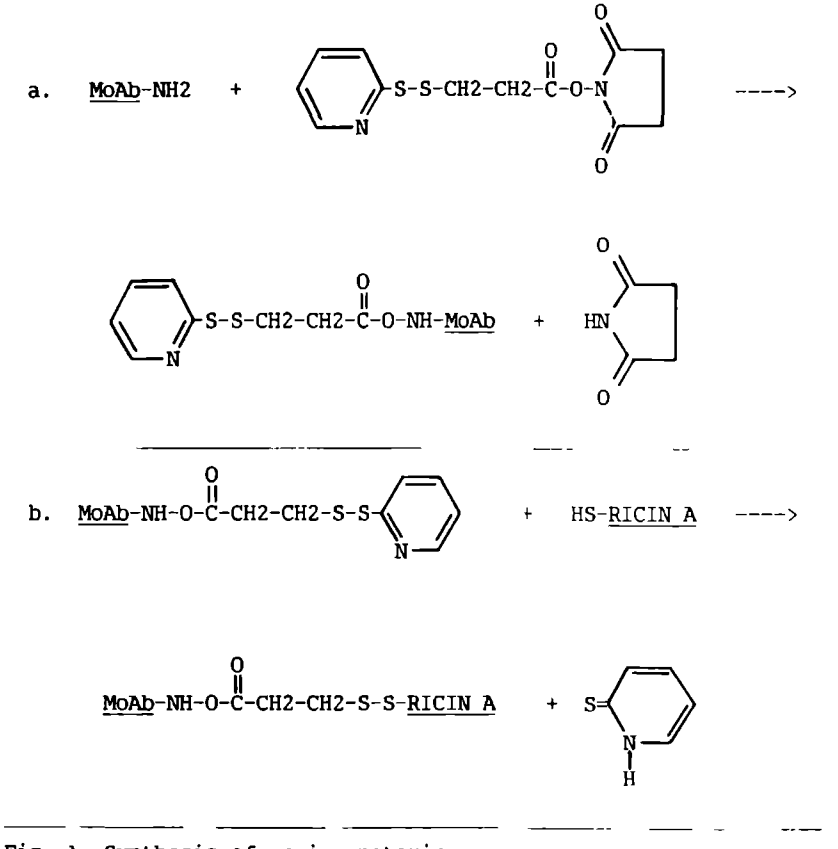

Fig. 1. Synthesis of an immunotoxin.

Fab- or $F(ab')_2$-fragments of the antibody are to be preferred to intact antibodies to circumvent the nonspecific binding of the Fc-receptor. The cytotoxicity of Fab-fragment containing ITs is significantly less than $F(ab')_2$-ITs or ITs of intact MoAbs [58,60]. The Fc-receptor binding does not substantially influence the specificity of the ITs in in vitro bone marrow purging, whereas after bone marrow infusion the concentration of the contaminating IT will be very low and the ITs are eliminated rapidly from the bloodstream [61].

In conclusion, the use of ITs composed of an intact MoAb and ricin A-chain, coupled with SPDP, appears to be an effective approach for the elimination of normal and malignant T cells in bone marrow. Though the before mentioned approaches will not be the ultimate solution for the treatment of leukemia, it will be a step forward on the road to a successful bone marrow transplantation. Moreover it opens the possibility for in vivo application to reduce the residual malignant cells after intensive radiochemotherapy.

4. AIM OF THE INVESTIGATIONS

In this thesis the use of ITs for the ex vivo elimination of malignant T cells in autologous bone marrow grafts and normal T cells in allogeneic bone marrow grafts was studied. The ITs were composed of monoclonal antibodies directed against T cell antigens conjugated to ricin A-chain.

Various parameters influencing the efficacy of ricin A ITs were analyzed. The suitability of different T cell antigens as target for ITs in relation to the quantitative expression of the antigens and their ability to internalize ITs was studied (Chapter 2).

The susceptibility of resting and activated normal human T cells to ITs in comparison with malignant T cell lines was studied. The effect of lysosomotropic agents on the cytotoxicity of the ITs was investigated (Chapter 3).

The efficacy of ITs to normal T cell subpopulations was compared with complement mediated cytotoxicity. The effect of the different anti-T cell ITs and MoAbs with complement on the natural killer cell activity was studied (Chapter 4).

The susceptibility of human malignant T cells to ITs was determined

as well as the optimal conditions for the ex vivo incubation of autologous bone marrow prior to reinfusion (Chapter 5).

The application of anti-T cell ITs for the purging of bone marrow in autologous BMT as treatment for acute T cell leukemia and lymphoma was analyzed in a clinical trial (Chapter 6).

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CHAPTER 2

RELATIONSHIP BETWEEN INTERNALIZATION AND CYTOTOXICITY OF RICIN A-CHAIN IMMUNOTOXINS.

F.W.M.B. Preijers, W.J.M. Tax, T.J.M. de Witte, Anke Janssen, Hélène v.d. Heijden, H. Vidal*, J.M.C. Wessels, P.J.A. Capel.

From Departments of Hematology and Nephrology, University Hospital Nijmegen, The Netherlands; *Department of Immunology, Centre de Recherches Clin Midy-Groupe Sanofi, Montpellier, France.

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ABSTRACT

Immunotoxins (IT) appear to vary considerably in their killing efficiency towards antigen positive cells. In order to unravel the mechanisms underlying these differences, the parameters responsible for these variations were studied. The efficacy of the monoclonal antibodies (MoAb) WT32 (CD3), OKT4 (CD4), T101 (CD5), WT1 (CD7), and WT82 (CD8) conjugated to ricin A-chain was expressed by the extent of protein synthesis inhibition of four leukaemic T cell lines (CEM, GH1, Jurkat, and HPB-ALL). Large differences in cytotoxicity were observed. Efficient inhibition of protein synthesis was seen with anti-CD3 IT, anti-CD5 IT, and anti-CD7 IT. In these cases the cytotoxicity was related to the antigen desity on the target cell membrane. Anti-CD4 IT inhibited poorly and anti-CD8 IT was ineffective, even on cell lines with a high expression of the corresponding antigen. When antigen density and cytotoxicity were plotted for all CD-antigens, no correlation could be found. Subsequently, internalization was studied with ^{125}Iodine-labelled antibodies. Anti-CD7 showed the fastest internalization rate, followed by anti-CD3 and anti-CD5. Anti-CD4 and anti-CD8 antibodies were respecively moderately and hardly internalized. When the absolute amount of internalized MoAb was calculated, a highly significant correlation with cytotoxicity was found. We conclude that the degree of antigen expression is not so important as the absolute amount of antibody internalized in predicting the efficacy of IT.

Immunotoxins (IT) exert a highly specific cytotoxicity to target cells by combining the specificity of an antibody and the cytotoxic properties of a toxin. The A-chain of the phytolectin ricin is an extremely potent irreversible inhibitor of protein synthesis by catalytical inactivation of the 60S ribosomal subunit (Endo & Tsurugi, 1987). The A-chain is only effective after translocation into the cytoplasm of the cell (Olsnes & Pihl, 1973), following an initial binding of the IT to the antigen and receptor-mediated endocytosis (Carrière et al, 1985). Ricin A-chain IT have slower kinetics than IT containing intact ricin (Vallera et al, 1984; Youle & Neville, 1982), possibly due to the capacity of the B-chain to increase the uptake of A-chain in the cytoplasm by facilitating the translocation of the A-chain from the endosome into the cytosol (McIntosh et al, 1983; Vitetta, 1986). However, the B-chain containing conjugates possess a lower specific cytotoxicity and therefore are less suitable for specific elimination of malignant cells (Skilleter et al, 1981). The potency of A-chain IT can be increased considerably by ammonium chloride (Casellas et al, 1984).

IT seem to be useful tools for the ex vivo elimination of leukaemic cells or mature T cells from bone marrow (Myers et al, 1984; Casellas et al, 1985; Derocq et al, 1987; Preijers et al, 1988a). Before clinical application of IT the conditions for optimal and selective elimination of malignant cells should be determined. Effective IT should satisfy the following conditions: high density of the target antigen (Myers et al, 1984; Laurent et al, 1986), intracellular stability of the A-chain enabling inhibition of protein synthesis (Olsnes & Pihl, 1983), and high internalization capacity of the target antigen.

In this paper we report that, although the desity of the target antigen on the cell membrane is a prerequisite for effectiveness of IT, the absolute amount of antibody internalized shows the best correlation with cytotoxicity of the different IT directed against T-cell differentiation antigens.

MATERIALS AND METHODS

Cell lines

The used human T-ALL cell lines (CEM, GH1, Jurkat, and HPB-ALL) were cultured in RPMI 1640 (Flow Labs, Irvine, Scotland), supplemented with 5% heat inactivated fetal calf serum (Flow Labs), 2 mM glutamine, 1 mM sodium pyruvate, and gentamycine (50 µg/ml) at 37°C in a humidified atmosphere with 5% CO2 in air.

Monoclonal antibodies

Murine anti-human T cell MoAb: WT1, anti-CD7 (Tax et al, 1984b); WT32, anti-CD3 (Tax et al, 1983); WT82, anti-CD8 (Tax et al, 1984a); T101, anti-CD5 (Royston et al, 1980); and OKT4, anti-CD4 (Kung et al, 1979) were purified using Staphylococcus aureus protein A coupled to Sepharose (Pharmacia Ltd, Uppsala, Sweden).

Immunotoxins

Ricin A-chain was kindly provided by Dr F.K.Jansen (Centre de Recherches Clin Midy-Group Sanofi, Montpellier, France). MoAb were coupled to ricin A by N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP, Pharmacia) as described previously (Preijers et al, 1988a). Final products were assayed by sodium dodecyl sulphate (SDS) gel electropheresis to determine the molecular weight. The molar ratio of A-chain and antibody were determined by radioimmunoassay and appeared to vary between 1 and 1.8. By flow cytometry (FCM) and enzyme-linked immunosorbent assay (ELISA; Tax et al, 1984b) it was demonstrated that the antibody binding activity was preserved after conjugation. The nonspecific cytotoxicity of all conjugates was tested on cell lines which did not express the relevant antigen. T101-ricin A was kindly donated by Dr P. Casellas (Centre de Recherches Clin Midy).

Cytotoxicity of IT to cell lines

Cytotoxicity assays were carried out in 96-well U-bottom plates (Costar, Cambridge, Massachusetts) in triplicate. Each well containing 10^5 cells in culture medium was supplemented with varying concentrations of ricin A-chain conjugates with or without 6 mM NH_4Cl in a final volume of 200 µl. The cells were incubated for 24 hrs at 37°C in a humidified

incubator with 5% CO2 in air, followed by a labelling for 24 hrs with 0.5 µCi ^{3}H-leucine (TRK510, Amersham International plc, England). Cells were harvested and radioactivity was counted. The incorporation of ^{3}H-leucine in the presence of 1 mM cycloheximide was used as background value. The cytotoxicity was expressed as percentage inhibition of ^{3}H-leucine incorporation of untreated cells corrected for the background.

Antigen density on the cell membrane

The expression of antigen was determined by MoAb labelled with ^{125}Iodine (Amersham, Buckinghamshire, England) using the chloramine T method (Hunter, 1973). The concentration and specific activity of the labelled MoAb were determined by radioimmunoassay and binding assay with limiting quantities of labelled antibody. Binding assays to determine the saturation level of MoAb were carried out at 4°C to avoid internalization of the bound MoAb. The maximal number of binding sites per cell was calculated after incubation of 10^{6} cells for 1 hr.

Determination of internalization of antibodies

The internalization of antibodies was determined as decribed previously (Tax et al, 1987). Briefly, target cells were incubated with ^{125}I-MoAb for various times at respectively 37°C or 4°C and radioactivity was counted. The amount of internalized MoAb was determined after removing the cell bound MoAb by incubation with either pronase (WT1 and WT82) or buffer at pH2 (WT32, OKT4 and T101). The percentage internalized antibody (%I) was calculated by the equation:

$$\%I = \left(\frac{A37^{\circ} - A4^{\circ}}{T4^{\circ}} \right) \times 100$$

T4° represents radioactivity after the first incubation at 4°C, and A37° and A4° the radioactivity of the samples after stripping the antibodies from the cell membrane, all corrected for nonspecific binding (always <250 cpm, <1% of the total bound label).

Table 1. Dose dependent cytotoxicity of IT to T-ALL cell lines and potentiating effect of NH_4CL.

IT	ID50* (M) CEM	GH1	Jurkat	HPB-ALL
WT32-ricin A	$>10^{-8}$	$>10^{-8}$	$5x10^{-10}$	$3x10^{-11}$
+NH_4CL	$>10^{-8}$	$>10^{-8}$	$2x10^{-10}$	$2x10^{-11}$
OKT4-ricin A	$3x10^{-9}$	$4x10^{-9}$	10^{-9}	$2x10^{-9}$
+NH_4Cl	$7x10^{-10}$	$7x10^{-11}$	10^{-10}	$2x10^{-10}$
T101-ricin A	$2x10^{-10}$	10^{-10}	$8x10^{-11}$	$3x10^{-11}$
+NH_4Cl	10^{-11}	10^{-12}	$2x10^{-11}$	$1.5x10^{-11}$
WT1-ricin A	$3x10^{-11}$	$3x10^{-12}$	$2x10^{-11}$	$7x10^{-9}$
+NH_4Cl	$8x10^{-12}$	$3x10^{-13}$	$3x10^{-12}$	10^{-9}
WT82-ricin A	$>10^{-8}$	$>10^{-8}$	$>10^{-8}$	$>10^{-8}$
+NH_4Cl	$>10^{-8}$	$>10^{-8}$	$5x10^{-9}$	10^{-8}

Cells were incubated with a range of IT concentrations with or without 6 mM ammonium chloride for 24 hrs followed by ^{3}H-leucine uptake for another 24 hrs. Data represent the mean of triplicate incubations of minimally five experiments.

*Concentration of IT which inhibited 50% of ^{3}H-leucine incorporation compared to untreated controls.

Cytotoxicity of anti-T cell IT on different cell lines

The cell lines CEM, GH1, Jurkat, and HPB-ALL were incubated with IT varying from 10^{-8} to 10^{-12} M with or without 6 mM ammonium chloride. The results, expressed as concentrations of IT inhibiting 50% of the protein synthesis (ID50), are given in Table 1. The level of protein synthesis

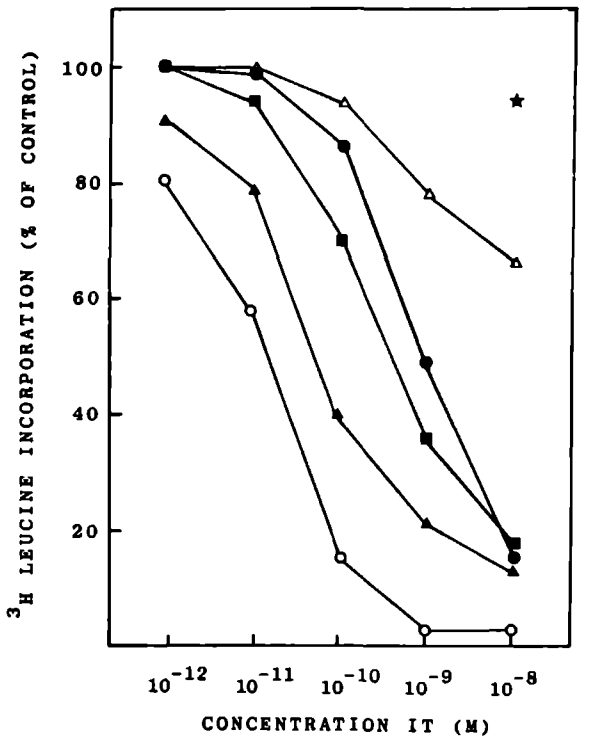

Fig. 1. Cytotoxicity of IT on Jurkat. Jurkat cells in culture medium were treated for 24 hr with different IT followed by 24 hr uptake of ^{3}H-leucine. Inhibition of protein synthesis is expressed as percentage ^{3}H-leucine incorporation of untreated controls. Each point represents the mean of triplicate incubation from minimally five experiments. Standard deviations were less than 10%.

(■) WT32-ricin A; (●) OKT4-ricin A; (▲) T101-ricin A; (○) WT1-ricinA; (△) WT82-ricin A; (★) free ricin A.

in untreated cells was similar for the different cell lines: GH1 (24285 ± 6532 cpm), HPB-ALL (23288 ± 5730 cpm), Jurkat (21729 ± 4531 cpm) and CEM (21665 ± 5023 cpm) (n=20). Ammonium chloride (6 mM) as such did not affect protein synthesis. Concentrations higher than 6 mM substantially influenced the protein synthesis.

Results of representative experiments with the cell line Jurkat expressing antigens for all the used IT, are depicted in Fig. 1. In this case WT82-ricin A did not induce a significant reduction of protein synthesis. The strongest decrease of ^{3}H-leucine uptake was found with WT1-ricin A, which was 50-fold more effective than OKT-ricin A.

Native ricin A-chain (10^{-8} M) gave only 6% reduction of protein synthesis in the absence and 11% reduction in the presence of 6 mM ammonium chloride for Jurkat. These values were the same or even lower for the other cell lines used (data not shown).

Toxicity of IT in relation to antigen density

The tested cell lines varied greatly in antigen density (Table 2). CEM showed no detectable expression of CD3 and CD8. The number of bound MoAb was compared with the extent of cytotoxicity of the IT of the different

Table 2. Antigen density of T cell lines.

		Antigen density (average no. of molecules bound per cell x 10^{-3} ± SD)			
CD	(MoAB)	CEM	GH1	Jurkat	HPB-ALL
CD3	(WT32)	0±0	6±2	73±9	185±12
CD4	(OKT4)	11±1	4±0	65±10	43±1
CD5	(T101)	25±1	32±0	52±6	64±3
CD7	(WT1)	63±3	175±25	106±5	20±1
CD8	(WT82)	0±0	16±2	141±8	75±0

SD = standard deviation. Antigen density is measured by binding analysis of ^{125}I-MoAb at 4°C. Data represent the mean of three independent measurements.

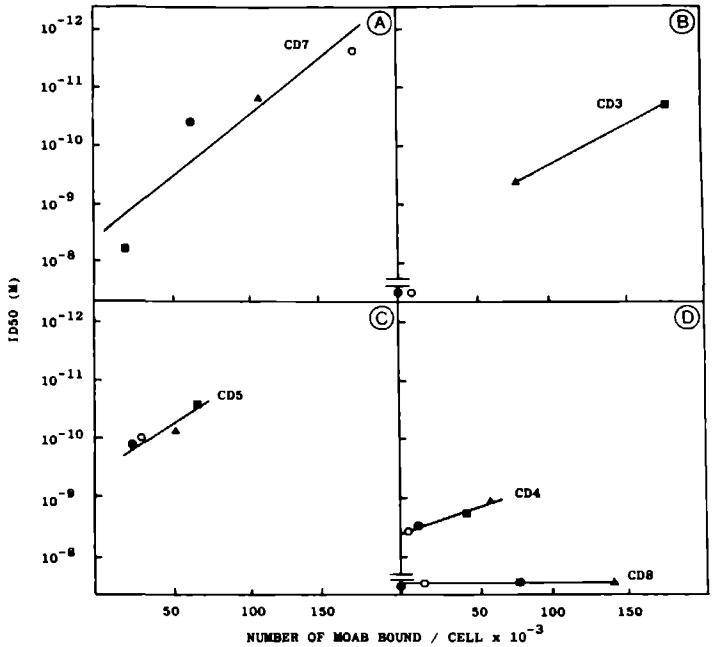

Fig. 2. Efficacy of IT in relation to antigen density of CD7 (A), CD3 (B), CD5 (C), CD4 (D), and CD8 (D) on the T-cell lines CEM (●), GH1 (○), Jurkat (▲), and HPB-ALL (■). Results are expressed as dose ID50 in relation to the maximal binding per cell of the concerning MoAb. Each ID50 value is the mean of triplicates of at least five independent experiments and has a standard deviation of less than 10%. MoAb binding was measured by ^{125}I-labelled MoAb in three independent experiments. Standard lines were obtained from linear regression analysis. Only ID50 values lower than 10^{-8} M ricin A IT were used for linear regression analysis.

MoAb (Fig. 2). Comparison of the ID50 of WT1-ricin A with the capacity of the cell lines to bind WT1, as shown in Fig 2A, showed a strong correlation ($p<0.01$) suggesting that the cytotoxic efficacy of this IT depends on the amount of antigen bound IT. Analysis of data of CD4 and CD5 antigens gave a significant correlation with respectively $p<0.05$ and

$p<0.01$ (Figs. 2C and 2D). The number of ID50's of WT32-ricin A (CD3; Fig. 2B) below 10^{-8} M were too small for linear regression evaluation, caused by a moderate or no expression of CD3 by respectively GH1 and CEM. Therefore a correlation could not reliably be calculated, but again the cell line with the highest density was most sensitive to WT32-ricin A. All ID50 values of WT82-ricin A (CD8) were higher than 10^{-8} M, even for cell lines with high antigen expression, indicating a very low efficacy of this IT.

When all data were pooled irrespective of CD antigen involved, no significant relationship was found between antigen density and ID50. This can also be derived from the variation in slope of the different linear regression lines in Fig 2. These results indicate that the antigen density is not the only factor that determines the cytotoxic efficacy of IT.

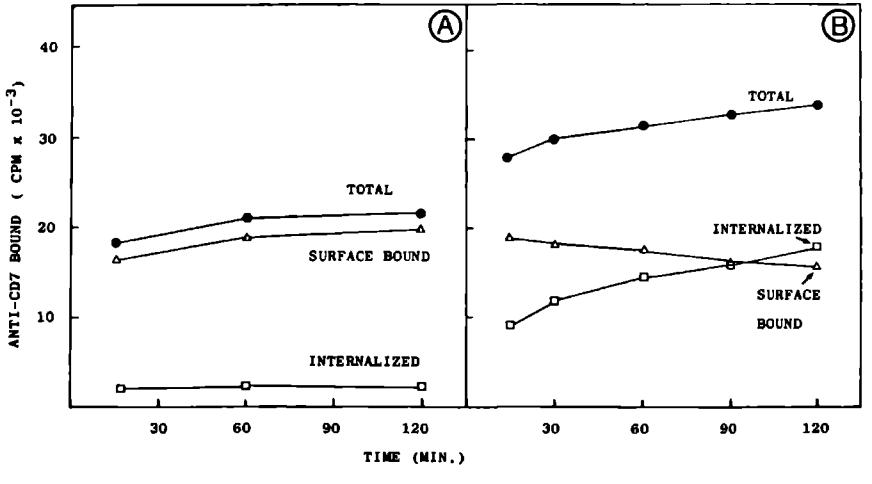

Fig. 3. Internalization of anti-CD7 MoAb (WT1). ^{125}I-labelled WT1 was incubated with CEM at 4°C (A) and 37°C (B) for various periods of time as indicated on the abscissa. Cell-associated ^{125}I-MoAb was counted. Internalized MoAb was determined after treating the cells with pronase as described in Materials and Methods.

Internalization of monoclonal antibodies

Because IT exert their function in the cytoplasm, we studied the capacity of the various antigens to internalize surface bound antibodies and ricin A-chain conjugated antibodies. The amount of antibody which was internalized within 1 hr after binding the specific antigen was examined with ^{125}I-labelled antibodies. An example of the binding and internalization kinetics is depicted in Fig. 3, showing an incubation of CEM cells with anti-CD7 (WT1). Similar internalization pattern was observed with ^{125}I-WT1-ricin A. The amount of surface bound antibodies at 4°C and 37°C is comparable. However, at 37°C the total amount of antibody has increased due to the internalization of antibody which only occurs to a significant degree at 37°C. The percentage internalized antibody after an incubation of 1 hr at 37°C was calculated for the used IT (Table 3). The highest percentage of internalized antibody was found for WT1, followed by WT32, and T101. The extent of internalization was

Table 3. Internalization of ^{125}I-labelled MoAb by T cell lines.

		Internalized ^{125}I-labelled MoAb (% ± SD)			
CD	(MoAB)	CEM	GH1	Jurkat	HPB-ALL
CD3	(WT32)	-*	30±2	69±5	43±10
CD4	(OKT4)	10±4	12±7	19±5	15±2
CD5	(T101)	34±3	38±6	38±1	35±1
CD7	(WT1)	76±10	90±3	72±2	67±9
CD8	(WT82)	-*	5±1	8±2	8±2

SD = standard deviation. Cells were treated with ^{125}I-MoAb for 1 hr at 37° C and 4° C. Radioactivity was counted and surface bound MoAb was removed as described in Materials and Methods. Percentage of internalized ^{125}I-MoAb was calculated from totally bound ^{125}I-MoAb. Data represent the mean of triplicate incubations for each test.

*No detectable expression of the antigen.

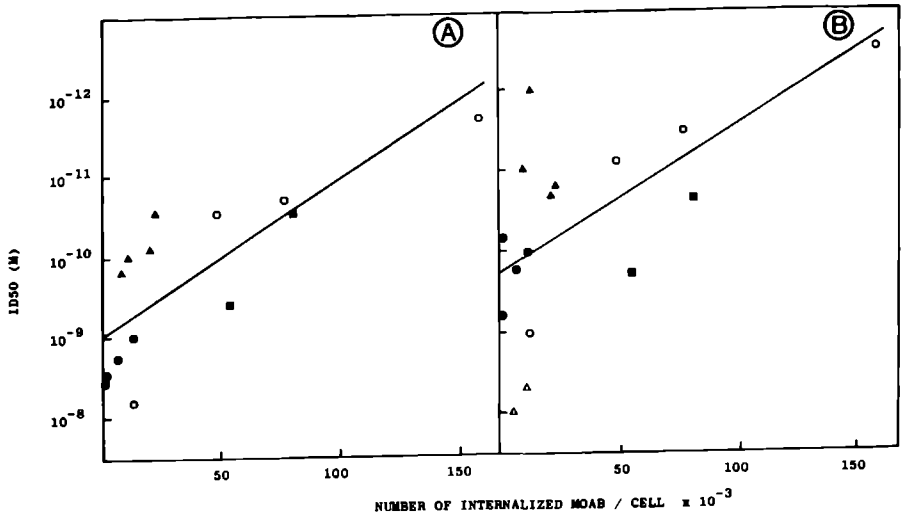

Fig. 4. Cytotoxicity of ricin A-chain IT in relation with the amount of internalized MoAb. Results are expressed as ID50 of ricin A-chain IT directed against CD3 (■), CD4 (●), CD5 (▲), CD7 (○), and CD8 (△) in the absence (A) or the presence (B) of 6 mM NH_4CL plotted against the total number of internalized MoAb, determined for the leukaemic T cell lines: CEM, GH1, Jurkat, and HPB-ALL. The standard line was obtained from linear regression analysis. Each single point represents the individual values of the ID50 and the total number of internalized MoAb of the described cell lines.

moderate for OKT4 and very low for WT82. Ammonium chloride did not influence the internalization (data not shown). Internalization patterns of IT were comparable with those of the corresponding free antibody showing that the conjugation procedure did not influence the internalization characteristics of the antibody.

Cytotoxic efficacy of IT in relation with the amount of internalized MoAb

From the data of Tables 2 and 3 the absolute amounts of internalized antibodies after an incubation of 1 hr under antigen saturating conditions were calculated. These values were compared with the ID50 values of the IT involved. A highly significant correlation ($r=0.78$ with $p<0.001$) was found (Fig. 4A). The number of internalized anti-CD8 and anti-CD4 antibodies was very low even for cell lines with high antigen density. This contrasted with the intracellular amount of MoAb which were well internalized, such as anti-CD3, anti-CD5, and anti-CD7. These results show that only after combining the antigen density and the internalization characteristics of this antigen, thus giving absolute figures for the amount of internalized IT, a relation with its ID50 becomes apparent. Incubation with ITs in the presence of 6 mM ammonium chloride decreased the ID50 about 5-fold without changing the correlation ($p<0.01$) between cytotoxicity and the number of internalized antibodies (Fig. 4B). The slope of the linear regression line remained unchanged.

DISCUSSION

Previous studies with WT1-ricin A (anti-CD7; Myers et al, 1984) and T101-ricin A (anti-CD5; Laurent et al, 1986) have suggested a direct relation between antigen density and cytotoxicity of IT for T cell lines. Comparable data with WT1 were also found for fresh leukaemic T cells (Preijers et al, 1988b). However, it is questionable whether this relation exists for all IT. In this report we showed that the cytotoxicity depends on the amount of internalized IT rather than on antigen density. Internalization of IT was determined after removing the cell surface bound MoAb from the target cell, whereas the number of binding sites on the cell surface was determined with saturating concentrations of ^{125}I-MoAb and with FCM (data not shown). The latter data appeared to be in accordance with observations of Poncelet & Carayon (1985).

We studied the relation between cytotoxicity, antigen density, and internalization of five ricin A-chain IT to leukaemic T cell lines. Our

results are in agreement with the data of Myers et al (1984) and Laurent et al (1986) concerning the relation between antigen density and cytotoxicity, but this relation only maintains for IT which were internalized in a high amount. For instance the T cell lines Jurkat and HPB-ALL with a high density of CD8, internalized a very low amount of WT82 which is in agreement with data of Meuer et al (1982). This indicates that anti-CD8 do not induce a decrease of the concerning cell surface antigen (modulation). The ID50 of WT82-ricin A was higher than 10^{-8} M which confirms the findings of Derocq et al (1987) and Till et al (1988).

CD4 antigen was also slightly internalized and comparable data were obtained from modulation experiments with anti-leu3a (Rinnooy Kan et al, 1983). We showed that OKT4-ricin A was poorly cytotoxic according the results of Derocq et al (1987).

The antigen with fastest internalization rate was CD7, followed by CD3 and CD5. We found that the relative degree of internalization of the CD7 antigen for all target cells studied was more than twice that of the the CD5 antigen (Table 3). In contrast, Leonard et al (1985) suggested, based on inhibition studied, that 3A1-ricin (anti-CD7) was less well internalized and therefore less effective than T101-ricin. Similar results were obtained by Stong et al (1985) using G3.7-ricin (anti-CD7) and T101-ricin, despite equivalent target cell binding. Obviously, besides the internalization rate, the intracellular handling of an IT is of importance for its cytotoxic effect.

The internalization of WT32 in 1 hr varied between 30% for GH1 and 69% for Jurkat comparable with findings of Tax et al (1987). Krangel (1987) demonstrated that the CD3-complex was partly internalized and recycled during a 3 hr period of examination, allowing a continuous internalization of IT. Rinnooy Kan et al (1983) showed complete modulation of CD3, but after a much longer incubation period in which degradation may occur.

Cytotoxicity of IT could be strongly enhanced by ammonium chloride. Ammonium chloride had no influence on the amount of internalized IT itself. The fact that lower amounts of IT were necessary for 50% protein synthesis inhibition (ID50: Fig. 4B) indicates that ammonium chloride most likely facilitates the transport of IT to the ribosomes by modifying the traffic between the different intracellular compartments

(Carrière et al, 1985). The average reduction of the ID50 was a factor 5, but differed considerably per antigen and per cell line. These results are also evidence for a different intracellular handling of ricin A per cell type. We conclude that, besides antigen density and an effective intracellular transport of ricin A to the site of action, the internalization of IT is the most important factor for a high efficacy.

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CHAPTER 3

DIFFERENT SUSCEPTIBILITIES OF NORMAL T CELLS AND T CELL LINES TO IMMUNOTOXINS.

F.W.M.B. Preijers, W.J.M. Tax, J.M.C. Wessels, P.J.A. Capel, T. de Witte and C. Haanen.

From Departments of Hematology and Nephrology, University Hospital Nijmegen, The Netherlands.

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ABSTRACT

In the context of ex vivo T cell elimination from bone marrow, the anti-T cell cytotoxic potential of immunotoxins (IT) prepared by conjugation of the monoclonal antibodies (MoAb) WT32 (CD3), T101 (CD5), and WT1 (CD7) to ricin A-chain was evaluated. The cytotoxicity of IT was based on protein synthesis inhibition in the human T cell lines: GH1, CEM, HPB-ALL, and Jurkat, and appeared closely related to the antigen density and internalization rate of the IT. Normal unstimulated T cells appeared to be rather insensitive to IT not due to a low antigen density or decreased internalization. The cytotoxicity of IT to T cells could be enhanced considerably by NH_4Cl. Treatment of T cells with a cocktail of IT (10^{-8} M) and 20 mM NH_4Cl resulted in a 5000-fold cytoreduction as measured by clonogenic assays of limiting T cell dilutions, whereas the haematopoietic progenitor cells remained unaltered. Stimulation of T cells with phytohaemagglutinin (PHA) prior to incubation with IT, considerably increased the sensitivity to IT treatment. Thus, normal T cells are less sensitive to anti-T cell IT than T cell lines and activated T cells. This suggests that a low protein synthesis is responsible for the resistance to IT. However, a high specific cytotoxicity of IT to normal T cells can be achieved in the presence of 20 mM ammonium chloride.

In vitro elimination of subpopulations from bone marrow has been attempted with monoclonal antibodies (MoAb). To increase the specific cytotoxicity MoAb have been conjugated to toxins such as the phytolectin ricin. These conjugates, immunotoxins (IT), appeared to be effective for the ex vivo elimination of malignant T cells from bone marrow [4,12,15,16,26,33]. The specificity of ricin IT has been improved by conjugation to the ricin A-chain after the removal of the ricin B-chain [18]. Although ricin A-chain IT are less potent than conjugates with intact ricin [31,35,38], their cytotoxicity can be increased considerably by lysosomotropic amines or carboxylic ionophores [3]. The cytotoxic potential of IT has been based mostly on the inhibition of protein synthesis or colony formation of cell lines. The magnitude of cytoreduction obtained by IT in cell lines varies from 1 log to more than 6 logs, and appears to be related to antigen density and antigen internalization capacity [20a], the actual intracellular toxin concentration at the site of action, the nature of the toxin, and the cell line being studied [4,12,15,16,21,26,33].

IT are also applied to remove immunocompetent T cells from donor bone marrow to prevent acute graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (BMT) [6,7]. Exposure of bone marrow cells to ricin A-chain IT in the presence of ammonium chloride considerably potentiates the elimination of T cells [11,14,24]. At present, only a few experimental data are available on the efficacy of ricin A-chain IT to normal T cells in comparison to cell lines in the absence of ammonium chloride [25,35].

This paper reports the cytotoxic effects of different ricin A-chain IT directed against CD3, CD5, and CD7 on normal T cells and T cell lines in relation to their antigen density and internalization capacity. The specific cytotoxic efficacy of IT was enhanced considerably by ammonium chloride. The cytotoxic effect of IT on haematopoietic progenitor cells remained negligible.

MATERIALS AND METHODS

Isolation of T lymphocytes and culturing of cell lines

Blood mononuclear cells were isolated from buffy coats by density centrifugation on 1.077 g/ml Ficoll-Hypaque (Pharmacia Ltd, Uppsala, Sweden) and resuspended in RPMI 1640 (Dutch modification; Flow Labs, Irvine, UK). T cells were enriched by removal of adherent cells by nylon wool filtration [20]. This cell population consisted of 90 ± 4% CD2/CD3 positive cells, as determined by flow cytometry (FCM). The cell suspension was supplemented with 10% heat inactivated fetal calf serum (FCS) (HyClone Labs, Logan, Utah), 2 mM glutamine, 1 mM sodium pyruvate and gentamycin (50 µg/ml).

In some experiments $2x10^6$ T cells/ml were stimulated with 50 µg/ml phytohaemagglutinin (PHA; Welcome Diagnostics, Dartford, UK) for 3 days at 37°C in a humidified atmosphere with 5% CO2 in air.

Human T-ALL cell lines (GH1, CEM, HPB-ALL, and Jurkat) were cultured in RPMI 1640 containing 5% FCS.

Monoclonal antibodies and preparation of immunotoxins

Murine anti-human T cell MoAb: WT1, anti-CD7 [30,36]; WT32, anti-CD3 [29]; and T101, anti-CD5 [23] were purified by means of Staphylococcus aureus protein A coupled to Sepharose (Pharmacia).

MoAb were treated with a 10-fold excess of succinimidyl 3-(2-pyridyldithio)propionate (SPDP; Pharmacia) [1]. The modified antibodies were incubated overnight with a 2.5-fold molar excess of ricin A-chain (kindly provided by Dr F.K. Jansen, Centre de Recherches Clin Midy, Montpellier, France) at 30°C. Free antibody and free A-chain were removed by Sephacryl S200 gel filtration. Conjugates were analysed by SDS-PAGE. The molar ratio of ricin A-chain and antibody, determined by radioimmunossay, appeared to vary between 1 and 1.8. The preservation of the antibody-binding activity after conjugation was proven by FCM and enzyme-linked immunosorbent assay (ELISA) [30]. T101-ricin A was kindly donated by Dr P. Casellas (Centre de Recherches Clin Midy).

Antigen density on the cell membrane

The maximum number of binding sites was determined by MoAb labelled with ^{125}Iodine (Amersham International, Amersham, Bucks, UK) using the

chloramine T method [9]. The concentration and specific activity of the ^{125}I-labelled MoAb were determined by radioimmunoassay and binding assay, respectively, with limiting quantities of labelled antibody. Binding assays to determine the saturating level of MoAb were performed at 4°C to avoid internalization of the bound MoAb. The maximum number of binding sites per cell was calculated after incubation for one hour.

Internalization of MoAb

The internalization of MoAb was determined as described previously [28]. The percentage internalized antibody (%I) was calculated by the equation:

$$\%I = \left(\frac{A37^\circ - A4^\circ}{T4^\circ} \right) \times 100.$$

T4° represents total bound radioactivity after incubation at 4°C, and A37° and A4° the radioactivity of the samples after stripping the MoAb from the cell membrane, all corrected for background values.

Cytotoxicity of IT to cell lines

Cytotoxicity assays were carried out in triplicate in 96-well U-bottomed plates (Costar, Cambridge, Mass, USA). Each well was filled with 10^5 cells in culture medium, supplemented with varying concentrations of IT to a final volume of 200 µl. Cells were incubated for 24 hrs followed by labelling for 24 hrs with 0.5 µCi ^{3}H-leucine (TRK510, Amersham). The cells were harvested and radioactivity was counted. The background incorporation was determined in the presence of 1 mM cycloheximide. The cytotoxicity was expressed as percentage inhibition of ^{3}H-leucine incorporation of untreated cells corrected for the background value.

Protein synthesis inhibition of normal T cells

T cells (10^6 cells/ml) were incubated with IT in the absence or presence of 20 mM NH_4Cl for 24 hrs, subsequently cultured in triplicate at a concentration of 10^5 cells per well with or without 50 µg/ml PHA for 48 hrs. Cells were labelled with ^{3}H-leucine, harvested and counted as described. The cytotoxicity was expressed as percentage inhibition of the ^{3}H-leucine incorporation of PHA-stimulated cells not treated with

IT and corrected for the background incorporation of unstimulated cells.

Cytotoxicity of ITs to PHA-stimulated T cells was determined as described for T cell lines. Unstimulated cells were cultured under the same conditions to determine the background incorporation.

Limiting dilution assay for T cells

T cells were isolated from fresh blood, incubated with ricin A-chain IT in the absence or presence of 20 mM ammonium chloride, washed, and suspended in RPMI 1640 supplemented with 15% FCS, 20% interleukin-2 (IL-2; Lymphocult, Biotest, Fairfield, N.J., USA), 15% PHA-leukocyte-conditioned medium (PHA-LCM), 50 µg/ml PHA, and 10 ng/ml phorbol 12-myristate 13-acetate (TPA). Untreated cells were diluted to 5, 10, 25, and 50 cells per well, treated cells to 50, 500, and 5000 cells per well in 96-well round-bottomed microtitre plates (200 µl per well). After 14 days of culture the proliferative lymphocytes in each well were counted. Cloning efficiency was determined by the minimum chi-square method from the Poisson distribution between cell number seeded per well and the logarithm of the percentage of negative wells [27].

Cytotoxicity of IT to bone marrow progenitor cells

Bone marrow was obtained after informed consent from patients undergoing cardiac surgery. Erythrocytes and mature granulocytes were removed by gradient centrifugation on Ficoll (1.077 g/ml). Cells (10^6/ml) were incubated with IT (10^{-8} M) with or without 20 mM ammonium chloride for 24 hrs. Cells were washed and the reduction of bone marrow progenitors was determined in clonogenic assays of granulocyte/macrophage colony-forming cells (CFU-GM) and erythroid burst-forming cells (BFU-E) as described previously [37].

RESULTS

Cytotoxicity of IT to T cells

Cytotoxicity of the anti-T cell IT WT32-ricin A, T101-ricin A, and WT1-ricin A was evaluated on T cells. Protein synthesis, determined immediately after incubation with a cocktail of these IT representing 10^{-7} M ricin A, was slightly reduced (less than 20%). Incubation with

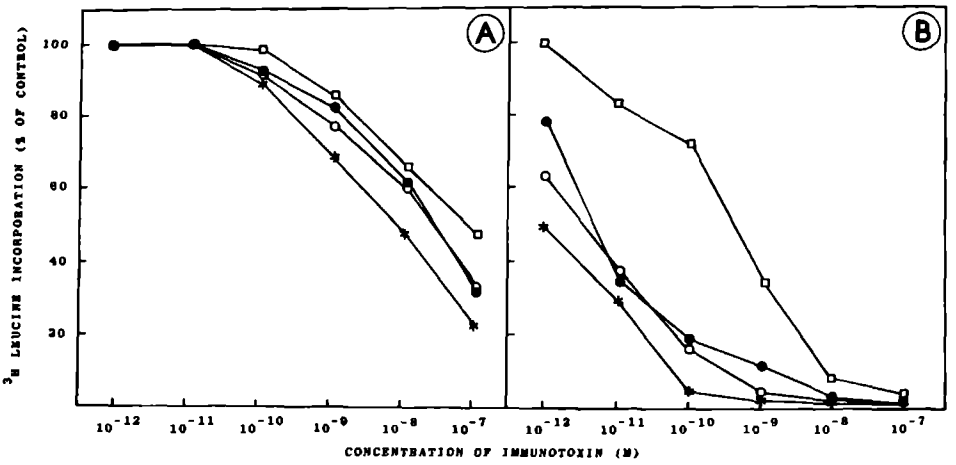

Fig. 1. Cytotoxicity of IT to normal T cells assessed by ^{3}H-leucine incorporation. T cells were pre-incubated with IT in the absence (A) or the presence (B) of 20 mM NH_4Cl, followed by stimulation with PHA and a 24 hrs uptake of ^{3}H-leucine. Inhibition of protein synthesis is expressed as percentage of ^{3}H-leucine incorporation of untreated controls. Each point represents the mean of triplicate incubations from at least four experiments. (□) WT32-ricin A; (○) T101-ricin A; (●) WT1-ricin A; (*) cocktail of WT32-, T101- and WT1-ricin A.

the individual IT resulted in a negligible inhibition (data not shown). These results should be interpreted with caution, in view of the low incorporation of ^{3}H-leucine by normal unstimulated T cells (10^5 cells incorporated maximally 1200 ± 400 cpm with an average background of 300 cpm; n=36).

When T cells were stimulated by PHA after treatment with IT, maximum leucine incorporation amounted 12,439 ± 3,665 cpm (n=16). The inhibition of protein synthesis occured at relative high concentrations of IT even with a cocktail of IT (Fig. 1A). The ID50 of the cocktail amounted 8×10^{-9} M (Table 1). In separate experiments it was shown that the

Table 1. Dose-dependent cytotoxicity of IT to normal T cells and potentiating effect of NH_4Cl.

	ID50* (M)	
	T cells	PHA-stimulated T cells
WT32-ricin A	10^{-7}	$4x10^{-9}$
+ NH_4Cl	$3x10^{-10}$	$7x10^{-11}$
T101-ricin A	$3x10^{-8}$	10^{-9}
+ NH_4Cl	$3x10^{-12}$	$7x10^{-13}$
WT1-ricin A	$3x10^{-8}$	$3x10^{-11}$
+ NH_4Cl	$3x10^{-12}$	$2x10^{-12}$
Cocktail of WT32-, T101-, WT1-ricin A	$8x10^{-9}$	$3x10^{-11}$
+ NH_4Cl	10^{-12}	$2x10^{-12}$

T cells or PHA-stimulated T cells were incubated with a range of IT concentrations with or without 20 mM NH_4Cl. Subsequently, T cells were cultured with PHA for 48 hrs, followed by ^3H-leucine uptake. PHA-stimulated T cells were labelled after IT treatment. The data represent the means of triplicate incubations of at least four experiments.
*Concentration of IT required for 50% inhibition of ^3H-leucine incorporation compared with untreated controls.

reduction of the proliferative responses to PHA by ricin A IT measured with ^{3}H-leucine incorporation was similar to reduction measured with ^{3}H-thymidine (data not shown).

The cytotoxicity of ITs was greatly enhanced by the addition of 20 mM ammonium chloride (Fig. 1B). Table 2 shows the enhancing effect of varying concentrations ammonium chloride on WT32-ricin A. Comparable results were found for T101-ricin A and WT1-ricin A. Concentrations of ammonium chloride higher than 25 mM resulted in substantial inhibition of haematopoietic progenitor cells. The potentiating effect of ammonium chloride appeared to depend on the IT used: WT32-ricin-A was potentiated with a factor 300 in contrast to a factor 10,000 for the other IT (Table 1). When the T cells were incubated with free ricin A-chain the protein

Table 2. Potentiating effect of NH_4Cl on cytotoxicity of WT32-ricin A.

10^{-8} M WT32-ricin A + NH_4Cl (mM)	^{3}H-leucine incorporation (cpm ± SD)
0	7905 ± 426 (70)*
6	5181 ± 651 (45)
10	2484 ± 203 (22)
15	1131 ± 191 (10)
20	565 ± 137 (5)
Untreated control	11290 ± 533 (100)
Untreated control + 20 mM NH_4Cl	11860 ± 560 (105)

10^5 T cells were incubated with 10^{-8} M WT32-ricin A in the presence of varying concentrations NH_4Cl, followed by stimulation with PHA. Protein synthesis inhibition was measured by ^{3}H-leucine incorporation in triplicate incubations.

*Numbers in parentheses represent the percentage of protein synthesis compared with untreated controls. Calculation were corrected for incorporation of unstimulated cells. The data represent the means ± standard deviation of five experiments.

synthesis was only slightly reduced (maximally 10%).

Limiting dilution assay for T cells

The number of T cells eliminated by IT treatment was assessed by clonogenic assays of limiting dilutions (Table 3). The cloning efficiency of untreated normal T cells was 1.7 ± 1.0 % (n=5). T cells were incubated with a concentration of IT (10^{-8} M) causing no non-specific cytotoxicity to haematopoietic progenitor cells. Wells with proliferative activity consisted of 95 ± 5 % CD2/CD3 positive cells as determined by FCM. The cocktail of the three IT caused 83% reduction in the number of clonogenic T cells, while incubation with the individual IT resulted in a lower reduction. Addition of 20 mM ammonium chloride during the incubation enhanced the cytotoxicity considerably (Table 3). The cocktail of the three IT reduced the number of proliferating cells by a factor of 5000 (99.98%). The reduction by the single IT was closely related to the percentage of antigen-positive cells as determined by FCM with the unconjugated MoAb.

Table 3. Depletion of T cells by IT in the absence or presence of NH_4Cl tested by limiting dilutions.

	Depletion of T cells (%)	
	$-NH_4Cl$	+20 mM NH_4Cl
-	0	6
WT32-ricin A	43	97.4
T101-ricin A	60	99.0
WT1-ricin A	65	97.5
Cocktail of WT32-, T101-, WT1-ricin A	83	99.98

Different concentrations of T cells were incubated with 10^{-8} M ricin A IT followed by a 14-day limiting dilution assay as described in Materials and Methods. The data represent the means of five experiments.

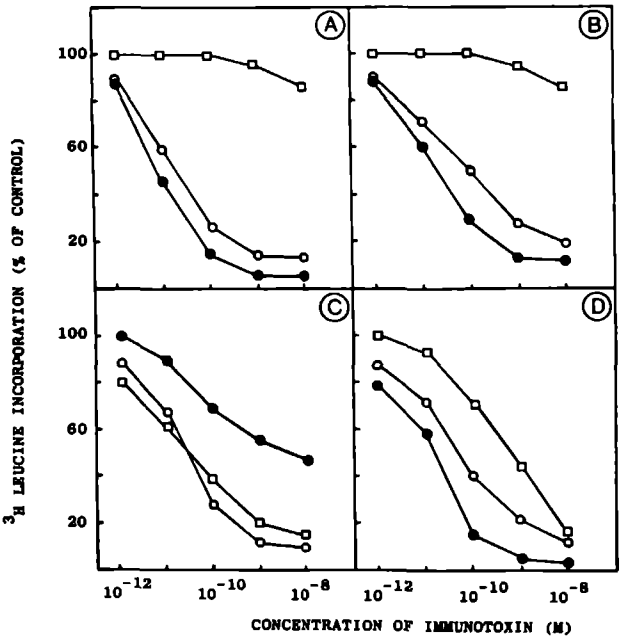

Fig. 2. Cytotoxicity of IT to T cell lines. The T cell lines GH1 (A), CEM (B), HPB-ALL (C), and Jurkat (D) were treated with different concentrations of WT32-ricin A (□), T101-ricin A (○), and WT1-ricin A (●), followed by a 24 hrs uptake of ^{3}H-leucine. Protein synthesis inhibition is expressed as percentage of ^{3}H-leucine incorporation of untreated controls. Each point represents the mean of triplicate incubations from at least five experiments.

Cytotoxicity of IT to cell lines

In order to show that the resistance of T cells towards IT was not caused by the ineffectiveness of the IT as such,the cytotoxicity of the ricin A-chain IT was tested on the T cell lines GH1, CEM, HPB-ALL, and Jurkat (Fig. 2). In contrast to normal T cells protein synthesis of all lines was reduced effectively in relation to the antigen density for the particular IT (Table 4). Effective cytotoxicity could also be shown by the fast kinetics of protein synthesis inhibition in all cell lines with a high antigen density for the particular IT. Inhibition of

Table 4. Antigen density of T cell lines and T cells.

CD (MoAb)	Antigen density (average no. of molecules bound per cell x 10^{-3} ± SD)				
	GH1	CEM	HPB-ALL	Jurkat	T cells
CD3 (WT32)	6±2	0±0	185±12	73±9	56±13
CD5 (T101)	32±0	25±1	64±3	52±6	22±1
CD7 (WT1)	175±25	63±3	20±1	106±5	48±7

Antigen density is measured by binding analysis of ^{125}Iodine-labelled MoAb at 4°C. The data represent the means ± standard deviation of three independent measurements.

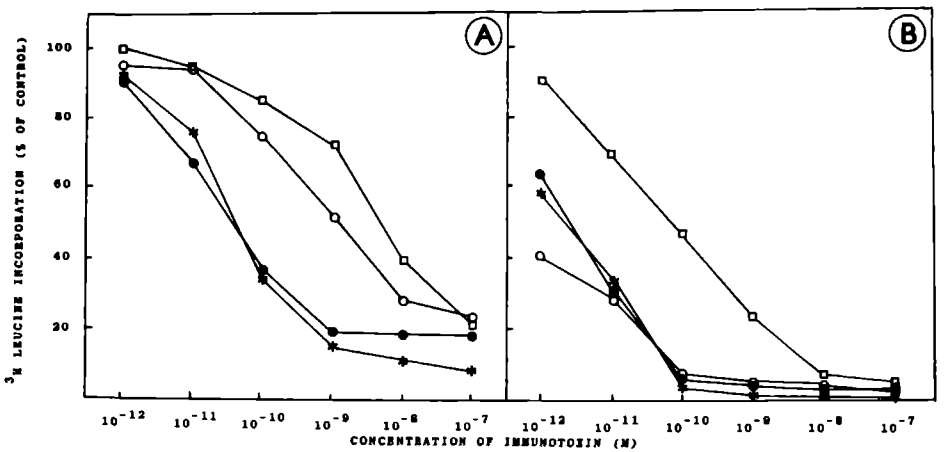

Fig. 3. Cytotoxicity of IT to PHA-stimulated T cells. Normal T cells were stimulated by PHA and incubated with IT in the absence (A) or the presence (B) of 20 mM NH_4Cl, followed by a 24 hrs ^{3}H-leucine incorporation. Inhibition of protein synthesis is expressed as percentage of ^{3}H-leucine incorporation of untreated controls. Each point represents the mean of triplicate incubations from at least four experiments.
(□) WT32-ricin A; (○) T101-ricin A; (●) WT1-ricin A;
(*) cocktail of WT32-, T101- and WT1-ricin A.

50% of the ^{3}H-leucine incorporation by 10^{-8} M WT1-ricin A in GH1 was achieved within 1 hr, by T101-ricin A in Jurkat within 3 hrs, and by WT32-ricin A in HPB-ALL within 5 hrs.

Antigen density and internalization of CD antigens on T cells and T cell lines

Comparison of the average antigen density on T cells and T cell lines (Table 4) did not explain the different sensitivities of the cell populations. CEM and GH1 expressed CD5 in similar amounts to normal T cells, but identical protein synthesis inhibition was obtained at 300- and 1000-fold lower concentrations of T101-ricin A, respectively.

The ability of normal T cells to internalize antibody was comparable with the internalization capacity of T cell lines (F. Preijers et al., unpublished observations). The percentages of internalization into T cells were 46% for WT32, 34% for T101 and 74% for WT1. The relative lower sensitivity of normal T cells compared to T cell lines is apparently not caused by a lower antigen density or a lower capacity to internalize the IT.

Cytotoxicity of IT to PHA-stimulated T cells

When T cells were stimulated with PHA prior to incubation with IT, higher cytotoxicity to T cells could be obtained, varying between 25-fold for WT32-ricin A, 30-fold for T101-ricin A, and 1000-fold for WT1-ricin A (Fig. 3A and Table 1). When the incubation with IT was performed in the presence of 20 mM NH_4Cl, the killing of unstimulated and stimulated T cells became comparable (Figs. 1B and 3B).

Cytotoxicity of IT to bone marrow progenitor cells

To examine the inhibiting effect of IT on haematopoietic stem cells, bone marrow samples were cultured after treatment with 10^{-8} M IT with or without 20 mM ammonium chloride. The treatment of bone marrow cells with IT in the absence of NH_4Cl did not affect the plating efficiency of CFU-GM and BFU-E (Table 5). In the presence of 20 mM NH_4Cl only a slight reduction was observed.

Table 5. Influence of IT and NH_4Cl on haematopoietic progenitor cells.

Incubation of bone marrow cells	CFU-GM (% ± SD)	BFU-E (% ± SD)
WT32-ricin A	99 ± 9	99 ± 10
T101-ricin A	102 ± 4	94 ± 13
WT1-ricin A	102 ± 8	97 ± 14
Cocktail of WT32-, T101-, WT1-ricin A	94 ± 10	98 ± 15
NH_4Cl	88 ± 10	72 ± 10
WT32-ricin A + NH_4Cl	75 ± 6	79 ± 15
T101-ricin A + NH_4Cl	82 ± 13	63 ± 13
WT1-ricin A + NH_4Cl	87 ± 10	75 ± 12
Cocktail of WT32-, T101-, WT1-ricin A + NH_4Cl	72 ± 8	76 ± 8

10^6 Bone marrow cells were incubated with 10^{-8} M IT in the absence or presence of 20 mM NH_4Cl. Thereafter clonogenic assays CFU-GM and BFU-E were performed. Numbers in the table represent the mean percentages ± standard deviation of colonies of untreated controls in five experiments.

In this study we compared the cytotoxicity of three anti-T cell IT: WT32-ricin A (anti-CD3), T101-ricin A (anti-CD5), and WT1-ricin A (anti-CD7), separately or as a cocktail, to normal T cells and to T cell lines. Clonogenic assays and protein synthesis inhibition assays were used to measure the cytotoxic effects on the target cells.

The three IT, separately or as a cocktail, did not appear to be very cytoxic to normal T cells, whereas cytotoxicity to T cell lines was considerable and comparable to data reported by others [4,16]. The low cytotoxicity of IT to normal T cells observed by us confirmed the findings of other studies with anti-CD5-ricin A IT [11,24], but contrasted with the data of Martin et al. [14]. They observed that anti-CD3-ricin A IT at a concentration of 1.7×10^{-8} M inhibited the ^{3}H-leucine incorporation by 81%. In our hands even a cocktail of anti-CD3, anti-CD5, and anti-CD7 ricin A conjugates induced not more than 50% protein synthesis inhibition with 8×10^{-9} M IT. Recently we have demonstrated a relationship between cytotoxicity of IT and the amount of internalized ITs into T cell lines [20a]. In this latter study we were able to show that internalization of IT into T cell lines and into normal T cells was equal. Therefore, it has to be concluded that the low susceptibility of normal T cells to IT must be due to an intrinsic property of these cells and not to a different internalization of the IT. This hypothesis is supported by the observation that stimulation of normal T cells by PHA prior to incubation with IT increased the susceptibility considerably. Since PHA activates protein synthesis the low level of protein synthesis in unstimulated T cells may be responsible for the limited sensitivity to IT.

When 20 mM ammonium chloride was added in combination with the IT, a high cytotoxicity to unstimulated T cells was observed. This cytotoxicity is in agreement with data from other investigators with ricin A IT [10,11,14,21,24] and comparable to the cytotoxicity of intact ricin IT [12,22,32,34]. The enhancement by ammonium chloride was similar for stimulated and unstimulated T cells. The cocktail showed better and more consistent cytotoxicity than the individual IT. Ammonium chloride did not influence the internalization process of the IT [2]. Ammonium chloride does, however, affect several intracellular processes related

to the handling of internalized IT. Figura et al. [5] found, after addition of ammonium chloride, a strong increase in the amount of newly synthesized lysosomal enzymes released from the cell in proportion to the amount delivered to the lysosomes. Furthermore, ammonium chloride can affect the activity of lysosomal enzymes by increasing the lysosomal pH [17]. In addition, the delivery of the IT to the lysosomes appeared to be delayed in the presence of ammonium chloride, which may decrease the lysosomal degradation of ricin A [2,21].

Our results show that the anti-T cell IT WT32-ricin A, T101-ricin A, and WT1-ricin A in a concentration of 10^{-8} M in the presence of 20 mM NH_4Cl have no influence on haematopoietic progenitors (CFU-GM and BFU-E). Similar minor inhibitory effects of haematopoietic progenitor cells have been described by others [11,14,24], whereas in that concentration T cells are eliminated by a factor of more than 5000, enabling in vitro depletion of human T cells from bone marrow intended for transplantation. Further experiments have to be carried out to determine the best combination of IT for the optimal elimination of T cells without the introduction of early graft failure or graft rejection after initial haematopoietic reconstitution [13,19]. Besides, the CD3-negative large granular lymphocyte population, which possibly have an anti-leukaemic effect [8], may not be affected.

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CHAPTER 4

HUMAN T LYMPHOCYTE DIFFERENTIATION ANTIGENS AS TARGET FOR IMMUNOTOXINS OR COMPLEMENT-MEDIATED CYTOTOXICITY.

F.W.M.B. Preijers, T. de Witte, G.P.M. Rijke-Schilder*, W.J.M. Tax*, J.M.C. Wessels, C. Haanen and P.J.A. Capel**.

From Departments of Hematology and *Nephrology, University of Nijmegen, and **Department of Experimental Immunology, State University Utrecht, The Netherlands.

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ABSTRACT

Graft-versus-host disease (GVHD) after allogeneic bone marrow transplantation (BMT) is initiated by immunocompetent T cells present in the graft. Selective elimination of distinct T-cell subsets or a sufficient, but not complete T-cell depletion, might abolish severe GVHD without graft rejection and loss of the anti-tumour potential. In this study we analysed the efficacy of different monoclonal antibodies (MoAb) WT32 (CD3), OKT4 (CD4), T101 (CD5), WT1 (CD7), and WT82 (CD8) with respect to their cytotoxicity to T cells either as immunotoxin (IT) or in combination with complement. The cytotoxic potential was assessed by protein synthesis inhibition and clonogenic assays. The ricin A conjugated MoAb exerted only a minor effect on blood or bone marrow T cells, although they were highly inhibitory to T-cell lines. However, in the presence of 20 mM ammonium chloride IT directed against CD3, CD5, and CD7 were highly cytotoxic. IT directed against CD4 and CD8 were less effective, due to a low internalization. The complement-mediated cytotoxicity was efficient for all antigens used.

The natural killer (NK) activity, as measured by cytotoxicity to K562, was hardly depressed by anti-CD3, anti-CD4, anti-CD5, and anti-CD8, but was eliminated by anti-CD7. All procedures used had only a minimal effect on haematopoietic progenitors as measured by CFU-GM and BFU-E assays.

We concluded that, although the T-cell population can be eliminated with the combination of anti-CD3, anti-CD5, and anti-CD7 antibodies plus complement, IT with 20 mM NH_4Cl appear to kill higher amounts of T cells. Selective elimination of CD4- and CD8-positive cells is effectively obtained by MoAb with complement.

Graft-versus-host disease (GVHD) is one of the major causes of morbidity and mortality following allogeneic bone marrow transplantation (BMT). It occurs in 30-60% of patients receiving HLA-identical sibling bone marrow, with a fatal outcome in approximately 50% of severely affected patients [37,40]. Several studies in rodents [5,10,13,16] have provided evidence that immunologically competent mature T cells present in the graft are responsible for the initiation of acute GVHD. Removal of T cells from the transplant reduces and even frequently prevents GVHD in man [8,12,22,27,29,42]. Although the minimal amount of T cells responsible for GVHD is not yet known and therefore complete depletion of T cells is sometimes propagated, such a drastic approach may increase the risk of a relapse [1] and may result in a delayed reconstitution of immune functions and increases the risk of graft rejection [20,25]. Preferably, T cells must be depleted from the transplant without concomitant loss of the subpopulations with anti-leukaemic capacity [6,11,41]. Depletion of immune competent T lymphocytes can be achieved by various methods, e.g. counterflow centrifugation [42], E-rosetting with or without prior lectin separation [7,9,29] or by means of monoclonal antibodies (MoAb) and rabbit complement (RC) [12,15,20,22,27]. Although not all methods are specific for the elimination of subpopulations, they have been applied successfully to deplete T cells from donor bone marrow intended for BMT. More recently, immunotoxins (IT) have been utilized to eliminate T cells in vitro because of their highly selective and potent cytotoxicity. Several IT have been prepared by conjugation of the highly toxic phytolectin ricin or its toxic A-chain to MoAb [8,21,26,28,32,38,39]. Investigations to determine the maximum elimination of malignant T cells have demonstrated that ricin A IT in the presence of ammonium chloride [3] are very effective when the target cells possess a sufficient density of the particular antigen on the cell membrane [4,23] and have the capacity to internalize the IT into the cell (F. Preijers et al., unpublished observations).

Recently we showed that normal T cells had a low susceptibility to ricin A-chain IT directed against CD3, CD5 and CD7 compared with T cell lines. The efficacy could be improved by addition of 20 mM ammonium

chloride [26]. These findings underlined the importance of preclinical investigations to assess the conditions that lead to an optimal elimination of T cells by different cytotoxic agents. In the present study we compared the cytotoxic efficacy of ricin A-chain IT directed against CD3 (WT32), CD4 (OKT4), CD5 (T101), CD7 (WT1), and CD8 (WT82) on normal T cells with the cytotoxicity of the complement-mediated lysis by the same MoAb. Moreover, the influence of these cytotoxic agents on NK activity was studied.

MATERIALS AND METHODS

Isolation of T lymphocytes

Peripheral blood mononuclear cells were isolated from the buffy coat and enriched for T cells by nylon wool filtration as described previously [26]. The percentage of T cells in the obtained cell suspension was determined by flow cytometry (FCM). Cells were suspended in RPMI 1640 supplemented with 10% heat inactivated fetal calf serum (FCS) (HyClone Labs, Logan, Utah), 2 mM glutamine, 1 mM sodium pyruvate and gentamycin (50 µg/ml).

Bone marrow T cells were isolated from aspirated donor bone marrow intended for allogeneic bone marrow transplantation by centrifugation over Percoll (Pharmacia, Uppsala, Sweden) gradients and counterflow centrifugation, as described previously [42,43]. The percentage of T cells was determined by FCM.

Monoclonal antibodies, complement and synthesis of IT

Murine anti-human T cell MoAb: WT1, anti-CD7 [36]; WT32, anti-CD3 [35]; WT82, anti-CD8 [35]; OKT4, anti-CD4 [17]; and T101, anti-CD5 [30] were of the IgG2a isotype and complement fixing. MoAb were purified using Staphylococcus aureus protein A coupled to Sepharose (Pharmacia).

Serum of 4 to 6- week-old New Zealand-White rabbits was used as complement (RC) source. Sera of all rabbits were tested separately at specific and nonspecific lysis of mouse spleen cells and human T-cell lines. Sera with optimal cytotoxicity were pooled, snap froozen and stored at -70°C until use.

Purified ricin A was kindly provided by Dr F.K. Jansen (Centre de

Recherches Clin Midy-Group Sanofi, Montpellier, France). MoAb were coupled to ricin A-chain with SPDP (Pharmacia). T101-ricin A was kindly donated by Dr P. Casellas (Centre de Recherches Clin Midy). Free antibody and free A-chain were removed by Sephacryl S200 gel filtration. The molar ratio of the A-chain and the antibody appeared to vary between 1 and 1.8. By FCM and enzyme linked immunosorbent assay (ELISA) it was proven that the antibody-binding activity was preserved after conjugation. The non-specific cytotoxicity of all conjugates was tested on cell lines which did not express the relevant antigen.

IT- or complement-mediated cytotoxicity to T cells

Cytotoxicity of IT to T cells in the absence or presence of 20 mM NH_4Cl was determined by inhibition of ^3H-leucine incorporation after an incubation for 24 hrs and stimulation by phytohaemagglutinin (PHA) for 48 hrs as described previously [26]. In experiments to determine the time dependency of the cytotoxicity, cells were incubated for 1, 2, 3, or 5 hrs. To determine complement-mediated cytotoxicity, cells were incubated for 23 hrs to synchronize experiments, prior to preincubation with varying concentrations of MoAb plus 1:6 vol/vol RC for 1 hr. Incubation with RC for more than 1 hr caused non-specific cytotoxicity. The cytotoxicity was expressed as a percentage of the inhibition of the ^3H-leucine incorporation of PHA-stimulated cells not treated with IT, incubated with or without ammonium chloride, or MoAb and complement, corrected for the background value obtained from cells not stimulated with PHA.

Cytotoxicity to bone marrow T cells was determined by preincubation of 10^7 cells/ml in 50 ml culture flask (Costar) in culture medium. Cells were supplemented with varying concentrations of ricin A-chain IT with or without 20 mM ammonium chloride during 24 hrs, or with varying concentrations of MoAb and RC (1:6 vol/vol) for 1 hr after culturing for 23 hrs in culture medium. Protein synthesis inhibition was determined as described before.

Limiting dilution assay for T cells

Clonogenic assays of limiting T cell dilutions were performed as described previously [26]. Briefly, T cells were incubated with IT (10^{-8} M) with or without 20 mM NH_4Cl for 24 hrs, or with MoAb (10 µg/ml:

6.7x10^{-8} M) plus RC (1:6 vol/vol) for 1 hr at 37°C. Cells were washed and suspended in RPMI 1640 (DM) supplemented with 15% FCS, 20% vol/vol interleukin-2 (IL-2; Lymphocult, Biotest, Fairfield, NJ, USA), 15% PHA-leukocyte-conditioned medium (PHA-LCM), 50 µg/ml PHA, and 10 ng/ml phorbol 12-O-tetradecanoylphorbol-13-acetate (TPA) and seeded in 96-well round-bottom microtiter plates. After incubation for 14 days proliferative lymphocytes were scored. Cloning efficiency was determined by the minimum chi-square method from the Poisson distribution between cells seeded per well and the logarithm of the percentage of negative wells [34].

IT- or complement-mediated cytotoxicity to normal bone marrow progenitor cells

Bone marrow was obtained from patients undergoing cardiac surgery after informed consent. Erythrocytes and mature granulocytes were removed by centrifugation on a gradient of Ficoll-Hypaque (1.077 g/ml). Cells (10^6/ml) were incubated with IT or MoAb and RC as described for the limiting dilution assays. After this incubation cells were washed and the reduction in bone marrow progenitors was determined in clonogenic assays of granulocyte/macrophage colony-forming cells (CFU-GM) and erythroid burst-forming cells (BFU-E) as described previously [44].

Natural killer activity after T cell depletion

Lymphocytes were isolated from peripheral blood by Ficoll (1.077 g/ml) centrifugation. Cells in a concentration of 4x10^6/ml were incubated in sealed tubes with 10^{-8} M ricin A IT in the presence of 20 mM NH_4Cl for 2 hrs (see Results) or 10 µg/ml MoAb with RC for 1 hr. Subsequently, the cells were washed and incubated for 18 hrs with 500 units/ml recombinant IL-2 in culture medium to increase NK activity [18]. Then cells were washed and placed in 96-well U-bottomed plates in serial dilutions to yield an effector to target ratio of 50:1, 25:1, 12.5:1, and 6.25:1 in 100 µl culture medium. ^{51}Cr-labelled K562 blasts were added in a concentration of 10^4/100 µl. After 3.5 hrs of incubation at 37°C, the cell mixtures were centrifuged and radioactivity of 100 µl supernatant was measured.

Maximum release was determined from K562 cells treated in culture medium with 10 µl saponin. Spontaneous lysis was measured from K562

incubated with culture medium only. NK activity was expressed as percentage ^{51}Cr-release by NK-mediated lysis:

$$\% \ ^{51}Cr\text{-release} = \frac{^{51}Cr\text{-release by NK} - \text{spontaneous } ^{51}Cr\text{-release}}{^{51}Cr\text{-release max.} - \text{spontaneous } ^{51}Cr\text{-release}} \times 100$$

RESULTS

Peripheral blood T cell population

The enriched lymphocyte population was analyzed by FCM. The cell population obtained by Ficoll gradient showed two scatter clusters: the lymphocyte area consisting of 68% cells and the monocyte area consisting of 23% cells. This cell suspension contained 70% $CD2^+$ and 64% $CD3^+$ cells. After nylon wool filtration the lymphocyte area contained 91% of the nucleated cells. This cell population contained 90% $CD2^+$, 82% $CD3^+$, 92% $CD5^+$, and 79% $CD7^+$ cells with a T4/T8-ratio of 1.2. Nylon wool filtrated cells were used in all experiments with peripheral blood cells.

IT- or complement-mediated protein synthesis inhibition of peripheral blood T cells

Cytotoxicity of the anti-T cell MoAb WT32 (CD3), OKT4 (CD4), T101 (CD5), WT1 (CD7) and WT82 (CD8), conjugated with ricin A-chain, to blood T cells was compared with complement- mediated cytotoxicity of these MoAb. T cells were incubated with varying concentrations between 10^{-7} and 10^{-12} M of IT or MoAb with RC. Results of T-cell treatment with the highest concentration of IT that caused negligible non-specific cytotoxicity (10^{-8} M) or MoAb (10 µg/ml: 6.7×10^{-8} M) with RC are shown in Fig. 1. Ammonium chloride as such did not inhibit the protein synthesis, whereas incubation with RC resulted in a slight reduction (8%). Ricin A-chain conjugates did not effectively inhibit the protein synthesis, but addition of 20 mM ammonium chloride resulted in a strong enhancement of the cytotoxicity of anti-CD3, anti-CD5, and anti-CD7 IT. A cocktail of these IT almost completely inhibited the protein synthesis. The RC-mediated cytotoxicity of anti-CD3, anti-CD5, and anti-CD7 was comparable to that of the corresponding IT.

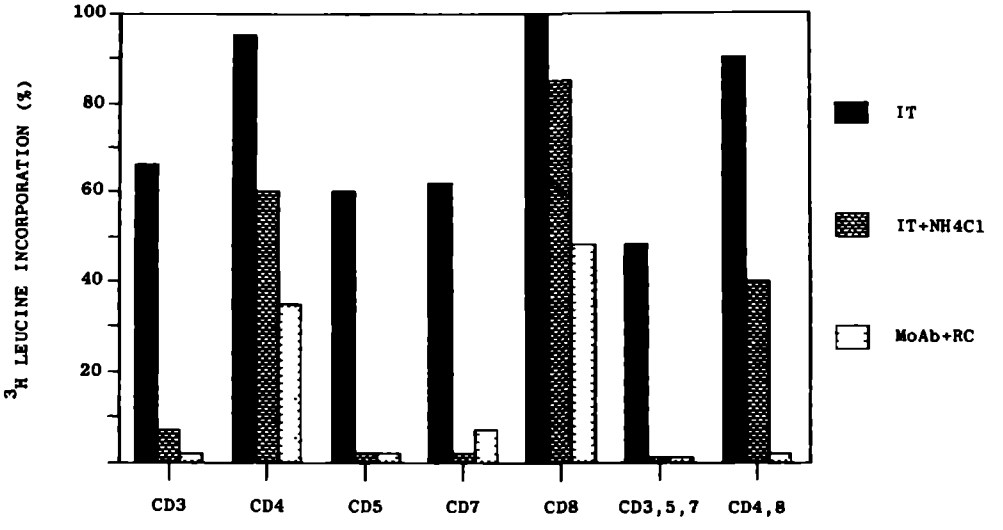

Fig. 1. Cytotoxicity of MoAb conjugated to ricin A or in combination with RC to blood T cells. T cells were incubated with 10^{-8} M IT in the absence or the presence of 20 mM NH_4Cl or with 10 µg/ml MoAb with 1:6 vol/vol RC, followed by stimulation with PHA and a 24 hrs incorporation of ^{3}H-leucine. Inhibition of protein synthesis is expressed as a percentage ^{3}H-leucine incorporation by untreated control cells. Each point represents the mean of triplicate incubations from minimum of five experiments and had a standard deviation of less than 10 %.

In contrast to the limited cytotoxicity of anti-CD4 and anti-CD8 ricin A IT with 20 mM NH_4Cl, treatment of cells with these MoAb and RC resulted in a protein synthesis inhibition according to the percentage of antigen positive T cells, while a cocktail of these MoAb plus RC inhibited the protein synthesis in nearly all T cells.

Limiting dilutions of peripheral blood T cells

The absolute depletion of T cells was determined by colony growth in limiting T-cell dilutions. The extent of T-cell depletion was determined from the cloning efficiency of native cells (Table 1). The cloning

Table 1. Cytotoxicity to T cells by IT with or without NH_4Cl or by MoAb with RC tested by limiting dilutions.

	Factor of T-cell reduction		
	IT	IT + NH_4Cl	MoAb + RC
-	1.0	1.1±0.1	1.3±0.7
CD3 (WT32)	1.8±1.2	38±14	61±30
CD4 (OKT4)	1.2±0.2	2.5±1.0	5.9±1.0
CD5 (T101)	2.5±1.0	100±35	252±86
CD7 (WT1)	2.8±0.4	40±14	31±13
CD8 (WT82)	1.0±0.0	1.3±0.3	2.0±0.5
Cocktail of WT32, T101, WT1	5.8±4.0	5000±670	167±51
Cocktail of OKT4, WT82	2.8±0.3	5.8±1.0	63±10

Different concentrations of T cells were preincubated with 10^{-8} M ricin A IT in the absence or the presence of 20 mM ammonium chloride or with 10 µg/ml MoAb plus RC, followed by a 14-day limiting dilution assay as described in Materials and Methods. Data represent the mean ± standard deviation of five experiments.

efficiency of untreated cells amounted 1.7 ± 1.0 % (n=5). These cells were cultured under identical conditions as treated cells. The delay in time, due to the preincubation explains the relatively low cloning efficiency. Wells with proliferating lymphocytes consisted of 95% $CD2^+/CD3^+$ cells, determined by FCM. Ricin A-chain IT in the absence of ammonium chloride caused only a slight reduction in the number of proliferative T cells, in contrast to appropriate IT in the presence of 20 mM ammonium chloride or MoAb with RC. Although the reduction by ricin A IT directed against CD3, CD5, and CD7 in the presence of 20 mM ammonium chloride was comparable with RC-mediated reduction by the MoAb,

the non-specific cytotoxicity of RC was higher than that caused by 20 mM ammonium chloride. A cocktail of the three IT directed against CD3, CD5, and CD7 with ammonium chloride appeared to reduce a higher percentage of the T-cell population (99.98%) than a combination of the free MoAb with RC (99.4%). These results show that a cocktail of ricin A-chain IT directed against CD3, CD5, and CD7 is very effective for the elimination of nearly all T cells.

ITs directed against CD4 and CD8 had hardly any effect. In contrast, $CD4^+$ and $CD8^+$ T cells could be fairly completely eliminated by the relevant MoAb with RC. The cocktail of these MoAb plus RC reduced 98.4% of the T-cell population (Table 1).

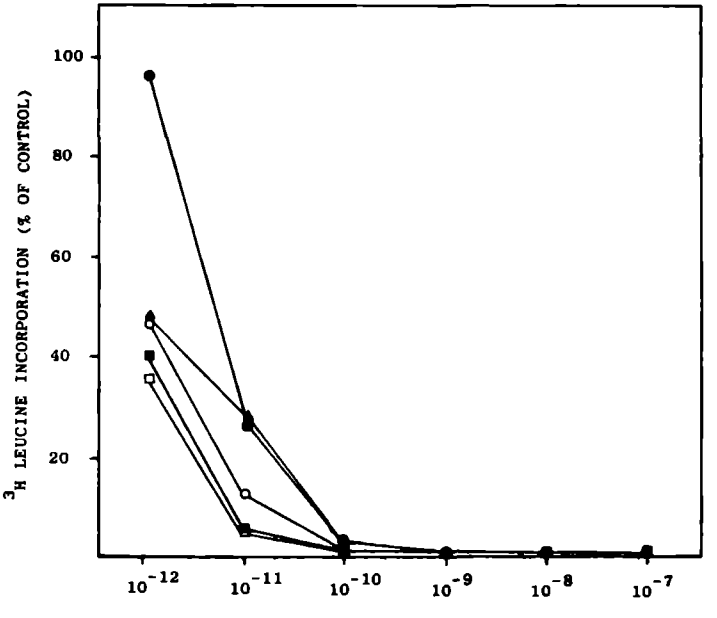

Fig. 2. Time dependent cytotoxicity of IT to blood T cells. T cells were incubated with varying concentrations of a cocktail of WT32-ricin A, T101-ricin A, and WT1-ricin A in the presence of 20 mM NH_4Cl for 1 (●), 2 (○), 3 (■), 5 (□), and 24 (▲) hrs, followed by stimulation with PHA and 24 hrs incorporation of ^{3}H-leucine. For additional legends see Fig. 1. Each point represents the mean of triplicate incubations from three experiments.

Influence of incubation period on cytotoxicity of IT

In our experimental setting the cocktail of WT32-ricin A, T101-ricin A and WT1-ricin A appeared to be more effective than the cocktail of MoAb with RC. However, cells were incubated for 24 hrs with IT in contrast to 1 hr with MoAb plus RC. In order to make both types of experiments comparable, the influence of incubation time on the cytotoxicity of IT was determined. Cells were treated for 1, 2, 3, or 5 hrs with the cocktail of the three IT with 20 mM NH_4Cl. An incubation of 2 hrs or more resulted in the same protein synthesis inhibition as effected by 24 hrs incubation (Fig. 2). These results suggest that an incubation for 2 hrs with a cocktail of IT is adequate for an optimal cytotoxicity.

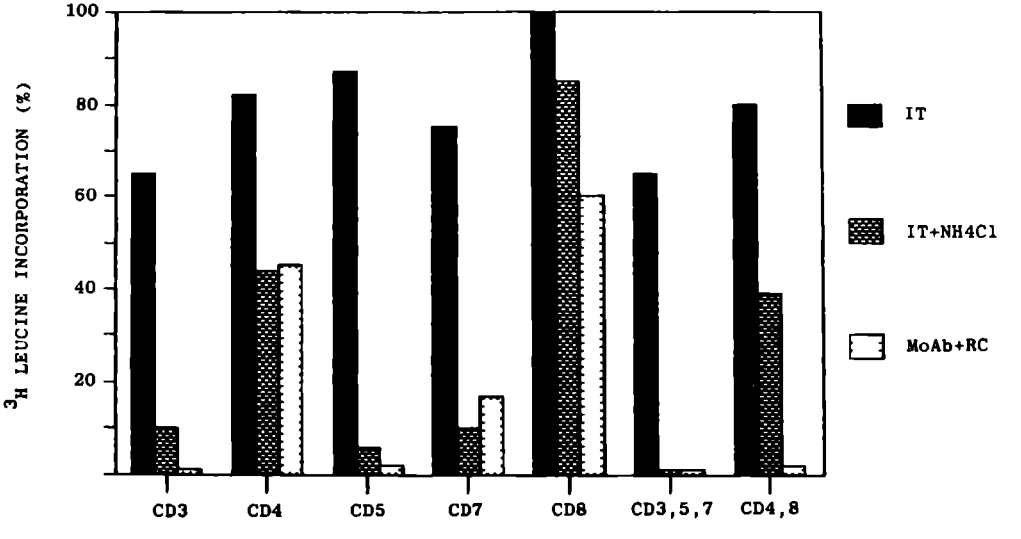

Fig. 3. Cytotoxicity of MoAb conjugated to ricin A or with RC to bone marrow T cells. T cells were isolated from bone marrow by elutriation and preincubated with 10^{-8} M IT with or without 20 mM NH_4Cl or with 10 µg/ml MoAb with 1:6 vol/vol RC in bulk culture of 10^7 cells/ml, followed by stimulation with PHA and 24 hrs incorporation of ^{3}H-leucine. For additional legends see Fig. 1.

Table 2. Influence of IT and NH_4Cl or MoAb with RC on hematopoietic progenitor cells.

Incubation of bone marrow cells	Recovery (% ± SD) CFU-GM	BFU-E
Cocktail of WT32-, T101-, WT1-ricin A	94 ± 10	98 ± 15
NH_4Cl	88 ± 10	72 ± 10
WT32-ricin A + NH_4Cl	75 ± 6	79 ± 15
T101-ricin A + NH_4Cl	82 ± 13	63 ± 13
WT1-ricin A + NH_4Cl	87 ± 10	75 ± 12
Cocktail of WT32-, T101-, WT1-ricin A + NH_4Cl	72 ± 8	76 ± 8
RC	98 ± 2	98 ± 5
WT32 + RC	88 ± 10	96 ± 5
OKT4 + RC	96 ± 4	98 ± 2
T101 + RC	91 ± 6	98 ± 3
WT1 + RC	91 ± 8	95 ± 5
WT82 + RC	90 ± 7	92 ± 8
Cocktail of WT32, T101 and WT82 + RC	90 ± 6	89 ± 8
Cocktail of OKT4 and WT82 + RC	99 ± 1	99 ± 3

10^6 Bone marrow cells were preincubated with 10^{-8} M IT in the absence or presence of 20 mM NH_4Cl for 24 hrs or with 10 µg/ml MoAb plus RC for 1 hr. Thereafter clonogenic assays CFU-GM and BFU-E were performed. Numbers in the table represent the mean percentage ± standard deviation of colonies scored with untreated controls in five experiments.

IT- or complement-mediated cytotoxicity to bone marrow T cells and haematopoietic progenitor cells

Bone marrow cells were treated on a large scale with IT and NH_4Cl or with MoAb and RC under conditions identical to donor bone marrow intended for transplantation. The bone marrow cell population contained 82% $CD2^+$, 65% $CD3^+$, 70% $CD5^+$, and 72% $CD7^+$ cells. Inhibition of protein synthesis in bone marrow T cells (Fig. 3) was comparable to that of blood T cells (Fig. 1). A cocktail of WT32-ricin A, T101-ricin A and, WT1-ricin A in the absence of ammonium chloride slightly reduced the protein synthesis (35%), while these IT in the presence of 20 mM ammonium chloride, similar to the cocktail of MoAb with RC, reduced the protein synthesis completely.

The non-specific IT- or complement-mediated cytotoxicity to haematopoietic progenitor cells was determined by clonogenic assays for CFU-GM and BFU-E. The treatment with IT or MoAb with RC hardly affected the plating efficiency of CFU-GM and BFU-E (Table 2). These results suggest that depletion of normal T cells from bone marrow by a selected cocktail of IT in the presence of 20 mM NH_4Cl or by MoAb plus RC is highly selective.

Influence of IT and MoAb plus RC on NK activity

The decrease of NK activity in the T-cell population by treatment with IT or MoAb with complement was tested on lymphocytes present in the low density fraction of a Ficoll separation to avoid any loss of NK-cell activity in the nylon wool column. The reduction of NK activity by varying effector to target ratios is shown in Figure 4. Preincubation with 20 mM ammonium chloride or 1:6 (vol/vol) RC alone did not influence the NK activity (NK activity of untreated cells is not shown). Anti-CD7, as IT or with complement, caused an almost complete reduction of the NK activity. Anti-CD3 and anti-CD5, as IT or with RC, did not measurably decrease the ^{51}Cr-release. Anti-CD4 and anti-CD8 with RC had no influence on the NK activity. These results suggest that the NK-cell activity is located in the CD3/CD4/CD5/CD8-negative and CD7-positive cell population.

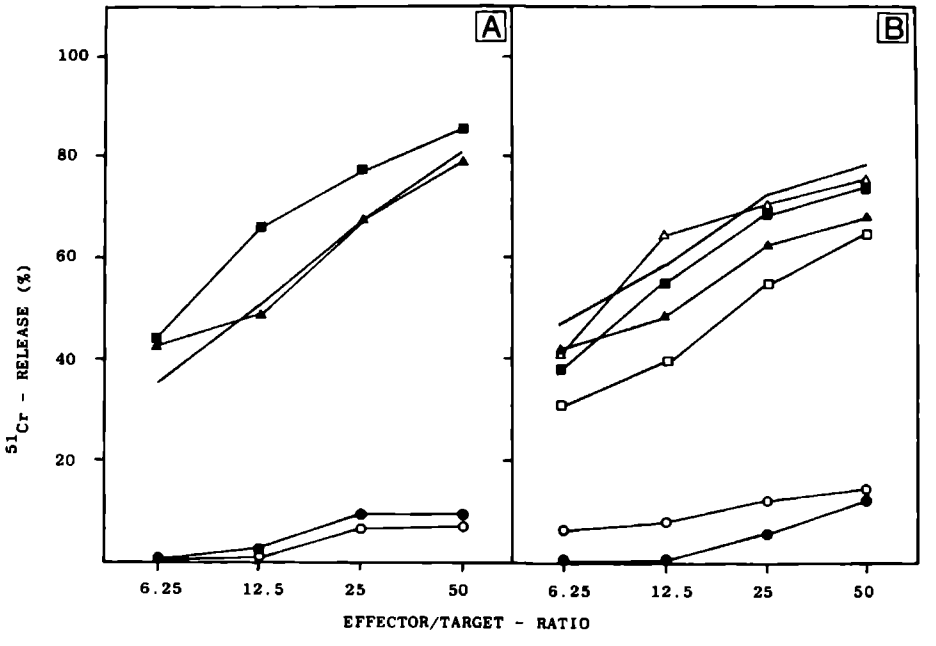

Fig. 4. Influence of IT and complement-mediated cytotoxicity of MoAb to NK activity. Blood T cells were preincubated with 10^{-8} M IT in the presence of 20 mM NH_4Cl (A) or with 10 µg/ml MoAb and 1:6 vol/vol RC (B) for 2 hrs and 1 hr respectively, prior to an incubation for 18 hrs with 500 U/ml recombinant IL-2. NK activity was based on the percentage of maximal ^{51}Cr-release of K562 and corrected for the spontaneous ^{51}Cr-release. The line without symbols represents the preincubation with NH_4Cl and RC, respectively. Values are the results of one representative experiment out of three.
(■) anti-CD3; (△) anti-CD4; (▲) anti-CD5; (●) anti-CD7; (□) anti-CD8; (○) anti-CD3-CD5-CD7.

DISCUSSION

Various methods can be used for the elimination of T cells from bone marrow. Monoclonal antibodies conjugated to ricin A-chain or MoAb in combination with RC appeared to be very efficient for this purpose [12,14,15,20-22,27,28]. In the present study we compared the cytotoxicity of ricin A conjugates and free MoAb in combination with RC directed against CD3, CD4, CD5, CD7, and CD8, separately or in combination. Cytotoxicity was determined by protein synthesis inhibition and by clonogenic assays. As found previously [26], ricin A conjugates appeared to be ineffective against normal T cells. Even a cocktail of ricin A IT directed against CD3, CD5 and CD7 removed only 84% of the T cells (Table 1). But in the presence of 20 mM ammonium chloride, these ITs eliminated all antigen-positive T cells. Ricin A IT and MoAb with RC killed T cells to comparable extent according to the findings of Martin et al. [21].

Ricin A IT directed against CD4 and CD8, however, showed a low cytotoxicity to normal T cells in the presence of ammonium chloride, due to a poor internalization of the target antigen (F. Preijers, unpublished observations). This minimal internalization may be related to the binding of IT to a specific epitope. Therefore, the efficacy of three different anti-CD8 ricin A IT was determined and a low cytotoxicity was found for all three IT (F. Preijers, unpublished observations). Low cytotoxicity of anti-CD8-ricin A to T cells was also found by Katz et al. [14] using a different anti-CD8 antibody. Furthermore, when we tested four other IT directed against the CD3/T cell receptor complex apart from WT32-ricin A, cytotoxicity of all tested IT was similar (F. Preijers, unpublished observations) and comparable to the data of others [21]. This again indicates that epitope-specificity is apparently not an important factor for the efficacy of an IT. With anti-CD4 or anti-CD8 antibodies, RC-mediated cytotoxicity was more effective than IT. A cocktail of these two MoAb plus RC killed 98.4% of the T cells, which is consistent with the percentage $CD4^+$ or $CD8^+$ cells in the cell suspension.

The individual IT directed against CD3, CD5, and CD7 in the presence of ammonium chloride exert a cytotoxicity comparable to the corresponding MoAb and RC. A cocktail of these ITs eliminated almost

all T cells (residual T cells: 0.02%) in contrast to 0.6% residual T cells after depletion with a cocktail of MoAb and RC. An effective cytotoxicity of anti-CD4 and anti-CD8 MoAb in a cocktail has only been observed for RC-mediated kill (this study and Refs. 12,19,20,27). Therefore, anti-CD4 and anti-CD8 IT should not be incorporated in a cocktail of anti-T cell IT.

A high degree of depletion of T-cell subpopulations seems to be necessary, particular for the prevention of GVHD after BMT of HLA-non-identical bone marrow. We determined the IT- and complement-mediated cytotoxicity to bone marrow T cells under circumstances comparable to those of donor bone marrow intended for transplantation; i.e. large volume and high cell concentration. The cytotoxicity to bone marrow T cells of both methods was similar to the results obtained with blood T cells, whereas only a marginal inhibition of hematopoietic progenitor cells was observed. Earlier experiments with IT were performed with an incubation time of 24 hrs. Since a shorter time would be a prerequisite for clinical applicability, we also studied shorter incubations with IT and found that a 2-hr incubation yields the same cytotoxicity.

Although the exact degree of T-cell depletion to prevent GVHD is unsettled, removal of excessively high numbers of T cells may result in a delayed reconstitution, failure of engraftment, or even rejection of the graft [25,31]. Moreover, the data of Van der Weiden, et al. [41] showed that transplant recipients who developed significant GVHD had a decreased risk of leukemia recurrence. The graft-versus-leukemia potential of the donor marrow will be compromised by complete T-cell depletion [1,22], resulting in a higher risk of leukaemia relapse. Although the precise cell subpopulation responsible for the anti-leukaemia effect is unknown, NK cells may contribute to this effect. Therefore, selective elimination of T-cell subpopulations responsible for GVHD without a loss of NK activity may improve the outcome after transplantation. We assessed the reduction of NK activity by the various combinations of IT and MoAb with RC. Anti-CD3 and anti-CD5 did not inhibit the NK activity, confirming the findings of Uckun et al. [38]. Moreover, no inhibition of NK activity was found with anti-CD4 and anti-CD8, whereas anti-CD7 inhibited NK cells effectively. These findings suggest that the NK population is CD3-negative, which may imply that this population consists of large granular lymphocytes (LGL) [2].

Incubation with high concentrations of IL-2 alone can predominantly activate a LGL population with CD3/CD4/CD8-negative and CD16/CD25-positive phenotype, with a minimal stimulation of T cells [24,33].

We conclude that ricin A-chain conjugates directed against CD3, CD5, and CD7, in a cocktail or separately, are very effective for the elimination of all T-cell subpopulations. However, the presence of anti-CD7 IT results also in the elimination of NK-cell activity in bone marrow intended for transplantation. These findings suggest that anti-CD7 should be omitted from cocktails intended to deplete donor bone marrow to prevent GVHD in order to maintain cells with potential anti-leukaemic activity in the graft. Besides, not all T-cell antigens represent targets through which effective kill can be achieved by ricin A IT due to the low internalization of some antigens (CD4 and CD8). These T-cell subpopulations can selectively be eliminated by MoAb and RC. Selective elimination of T lymphocytes with GVH reactivity, and preservation of populations with anti-leukaemic activity may improve the clinical outcome after allogeneic BMT.

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CHAPTER 5

CYTOTOXIC POTENTIAL OF ANTI-CD7 IMMUNOTOXIN (WT1-RICIN A) TO PURGE EX VIVO MALIGNANT T CELLS IN BONE MARROW.

F.W.M.B. Preijers, T. de Witte, J.M.C. Wessels, J.P.P. Meyerink, C. Haanen and P.J.A. Capel*.

From Department of Haematology, University Hospital Nijmegen, and *Department of Experimental Immunology, State University Utrecht, The Netherlands.

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ABSTRACT

With the perspective of bone marrow purging in autologous transplantation, we investigated the cytotoxicity of the anti-T cell immunotoxin (IT) WT1-ricin A (anti-CD7) to malignant T cells obtained from patients with T cell acute lymphoblastic leukaemia or lymphoma. The cytotoxic efficacy of IT was based on the extent of protein synthesis inhibition. Cytotoxicity of IT to malignant T cells showed a dependency on antigen density comparable to the T cell lines GH1, CEM, Jurkat, HSB-2 and HPB-ALL and was enhanced considerably in the presence of 6 mM ammonium chloride. The ultimate proof of cell kill can only be obtained from clonogenic assays, however culturing of malignant T cells was not feasible. Therefore these assays were performed with the cell line CEM that expresses comparable amounts of CD7 antigen as malignant T cells of most patients. More than 6-logs of CEM appeared to be eliminated after incubation with 10^{-8} M WT1-ricin A.

Immunotoxins are only effective after entering the target cell. The pattern of internalization of the IT was determined by means of ^{125}I-WT1. After internalization the CD7 antigen was re-expressed on the cell membrane. This enables a long incubation period resulting in an increased elimination of malignant T cells. Even after 16 hrs the IT was still accumulated intracellularly. This pattern of continuous uptake of IT was reflected in a gradually increasing cytotoxicity with incubation time. Effective bone marrow purging can be carried out without adverse effects on progenitor cells with 10^{-8} M WT1-ricin A. At that concentration the antibody binding capacity was saturated.

We showed that the protein synthesis inhibition in malignant T cells by WT1-ricin A is comparable to the inhibition in T cell lines and that high amounts of CEM cells can be killed. These data suggest that cell lines can be used to test the efficacy of IT to malignant T cells. WT1-ricin A appears to be very potent for the purging of autologous bone marrow from patients with T cell malignancies.

Autologous bone marrow transplantation (ABMT) is currently being investigated as a therapy for patients with acute leukaemia or lymphoma who do not have a HLA-matched sibling donor. Residual malignant cells contaminating the bone marrow of patients in complete remission, may be responsible for the high relapse rate after ABMT (Dicke et al, 1984; Santos & Kaizer, 1984; Gorin et al, 1986). Effective ex vivo purging of the bone marrow, however, may improve the therapeutic efficacy. Immunotoxins (IT) are considered to be very useful to eliminate selectively leukemic cells from the bone marrow. The efficacy depends on several cellular properties such as antigen density and internalization capacity, type of target cell, the nature of the toxin, and the incubation conditions (Casellas et al, 1985; Uckun et al, 1985; Press et al, 1986; Youle et al, 1986; Preijers et al, 1988a, b). Cytotoxicity of ricin A-chain IT can be enhanced considerably by lysosomotropic amines such as ammonium chloride or ionophores as monensin and nigericin (Casellas et al, 1984).

Before clinical applications can be instituted, the cytotoxic efficacy of the IT to the leukaemic cells of individual patients has to be determined to warrant an optimal elimination. In the absence of a reliable cloning method for leukaemic T cells, the cytotoxic efficacy of IT to clonogenic cells is usually tested by means of leukaemic human T cell lines. Since there is no evidence in literature that the cytotoxic efficacy of IT to leukaemic cells can be compared to cell lines, this study was performed. The anti-CD7 monoclonal antibody (MoAb) WT1 (Vodinelich et al, 1983) conjugated to ricin A, appeared to be a very potent inhibitor of protein synthesis of human T cell lines and offers a promising approach for the removal of malignant T cells from bone marrow in vitro without being harmful to haematopoietic progenitors (Myers et al, 1984; Preijers et al, 1988a, b).

In this report we describe the cytotoxicity of WT1-ricin A to leukaemic T cells, compared to T cell lines, and the optimal conditions for incubation based on the absolute amount of internalized MoAb. A relation was found between antigen density and the ID50 of WT1-ricin A for malignant T cells of most patients and appeared comparable to that previously observed in cell lines (Preijers et al, 1988a). WT1-ricin A

appeared effective for the ex vivo elimination of malignant T cells from bone marrow of patients without compromising the repopulation capacity of the autologous grafted bone marrow. Patients with a low density of CD7 on their malignant T cells tended to relapse earlier (to be published).

MATERIALS AND METHODS

Cell lines and malignant T cells

T cell acute lymphoblastic leukaemia (T-ALL) lines, strongly varying in CD7 expression, were selected: GH1, CEM, Jurkat, HSB-2 and HPB-ALL. Cells were cultured in RPMI 1640 supplemented with 5% heat inactivated fetal calf serum (FCS; HyClone Labs, Logan, Utah), 2 mM glutamine, 1 mM sodium pyruvate and gentamycin (50 µg/ml) in a humidified incubator with 5% CO2 in air at 37°C.

Malignant T cells were obtained from untreated patients with T-ALL or lymphoblastic lymphoma (T-LL). T-ALL cells were enriched from bone marrow by Ficoll (1.077 g/ml) centrifugation, whereas T-LL cells were isolated from lymph node tissue obtained by biopsy. Tissue was gently pressed through a 70 µm nylon filter, washed and centrifugated on Ficoll (Janssen et al, 1984). The isolated cell population of all patients consisted of more than 90% CD7-positive cells, as determined by flow cytometry (FCM). The cell suspensions contained comparable numbers of malignant cells as was proven by morphologic analysis. The cells were suspended in culture medium and used directly, or cryopreserved with 10% dimethylsulphoxide (DMSO) according to a computerized freezing programme with maintenance of their functional and growth potential (Van de Ouweland et al, 1982).

Immunotoxin

The murine anti-human T cell MoAb: WT1, anti-CD7 (Tax et al, 1984) was purified by means of Staphylococcus aureus protein A coupled to Sepharose (Pharmacia).

Ricin A was kindly provided by Dr F.K. Jansen, Centre de Recherches Clin Midy, Montpellier, France. WT1 was conjugated to ricin A by means of succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pharmacia) as

described elsewhere (Preijers et al, 1988a). The conjugated MoAb was disposed of free antibody and free A-chain by Sephacryl S200 gel filtration. The conjugate was analyzed by SDS-page gel electrophoresis. The molar ratio of ricin A-chain and antibody, determined by radioimmunoassay, appeared to vary between 1 and 1.8. The preservation of the antibody binding activity after conjugation was proven by FCM and enzyme linked immunosorbent assay (ELISA).

Antigen density and internalization

WT1 was labelled with ^{125}Iodine (Amersham International, Amersham Bucks., UK) using the chloramine T method (Hunter, 1973). The concentration and specific activity of the ^{125}I-labelled MoAb were determined by respectively radioimmunoassay and binding assay with limiting quantities of labelled antibody. The absolute number of binding sites per cell was determined as described previously (Preijers et al, 1988a).

The amount of internalized MoAb was determined as described (Tax et al, 1987). Briefly, 10^6 target cells in RPMI with 1% BSA were incubated with various concentrations ^{125}I-MoAb for 1 hr, or with fixed concentrations for various times at 37°C or 4°C in a final volume of 100 µl. The cells were washed, counted for total radioactivity, and incubated with 1 mg/ml pronase for 2 hrs at 4°C. Subsequently, the cells were washed and radioactivity was counted. The absolute amount of internalized MoAb was calculated from the pronase treated cells initially incubated at 37°C corrected for the pronase treated cells initially incubated at 4°C, and the concentration of ^{125}I-MoAb in the incubation medium. Binding at 37°C was determined by subtraction of the internalized radioactivity from the total radioactivity after incubation at 37°C.

Protein synthesis inhibition by WT1-ricin A

Cytotoxicity assays were carried out in triplicate in 96-wells U-bottomed plates (Costar, Cambridge, MS). Cell lines were incubated in culture medium and fresh or thawed cryopreserved leukaemic cells in EMEM without leucine (Flow Labs, Irvine, Scotland, UK) supplemented with 5% FCS, 2 mM glutamine, 1 mM sodium pyruvate and gentamycin (50 µg/ml). Each well was filled with 10^5 cells supplemented with varying

concentrations of IT in the absence or the presence of 6 mM NH_4Cl to a final volume of 200 µl. Cells were incubated for 24 hrs at 37°C in a humidified incubator with 5% CO2 in air, followed by 24 hrs incubation with 0.5 µCi ^{3}H-leucine (TRK510, Amersham). To enable kinetic studies cells were incubated for various periods of time with 10^{-8} M WT1-ricin A followed by a labelling for 1 hr with 1 µCi ^{3}H-leucine. Cells were harvested and radioactivity was counted. The cytotoxicity was expressed as percentage inhibition of ^{3}H-leucine incorporation of untreated cells corrected for the background value determined in the presence of 1 mM cycloheximide. No differences were observed between fresh and cryopreserved cells (data not shown).

Clonogenic assay

The clonogenicity of CEM cells was determined with the double agar layer cloning assay (Casellas et al, 1985). Cells in exponential phase of growth were incubated for 24 hrs in RPMI 1640 plus 10% FCS with concentrations of WT1-ricin A varying between 10^{-8} and 10^{-14} M in the absence or the presence of 6 mM NH_4Cl. The cells were washed and resuspended in cloning medium consisting of RPMI supplemented with 1 mM α-ketoglutarate (Sigma, St. Louis, USA), 1 mM sodium oxaloacetate (Sigma), 5% FCS, and 10% heat-inactivated horse serum. Numbers of cells, varying between 10^7 and 10^6, were plated in 2.5 ml cloning medium with 0.3% agarose (type VII, Sigma) upon a bottom layer of 2.5 ml of the same medium in 60 mm culture dishes (Costar). The cells were incubated at 37°C for 20 days and colonies were scored. The cloning frequency of IT treated cells was determined by means of the plating efficiency of untreated cells that were seeded from 1 to 100 cells/well. Absolute numbers of surviving cells were calculated from the number of colonies, the dilution of cells and the cloning frequency.

Protein synthesis inhibition of T cell lines

Cytotoxic potency of WT1-ricin A in the presence of 6 mM ammonium chloride was tested on T cell lines: GH1, CEM, Jurkat, HSB-2 and HPB-ALL and based on protein synthesis inhibition. Ammonium chloride as such did not affect protein synthesis. The antigen density of the cell lines, which varied from 20 kD (HPB-ALL) to 175 kD (GH1), was directly proportional to the cytotoxicity of the IT as determined by linear regression analysis ($p<0.01$; Fig 1A).

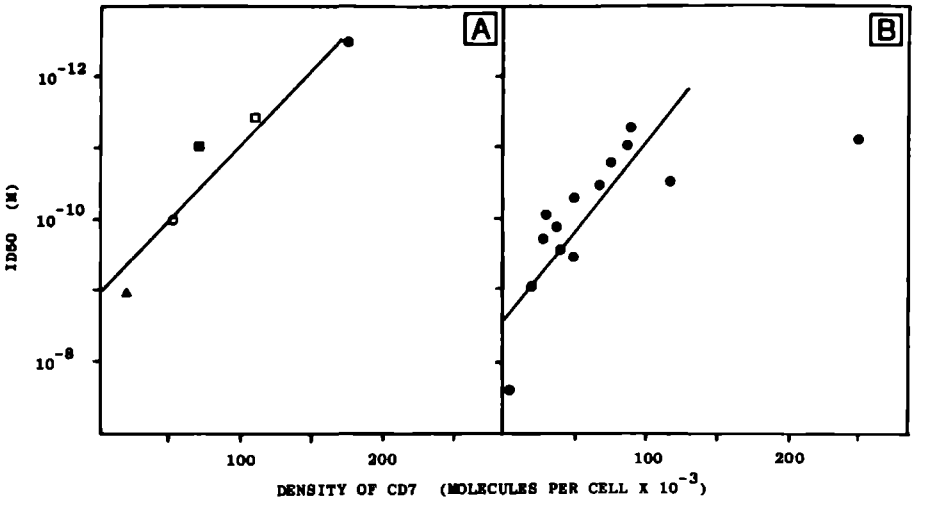

Fig. 1. Efficacy of WT1-ricin A in relation to antigen density of CD7 on T cell lines: GH1 (●), Jurkat (□), CEM (■), HSB-2 (○), and HPB-ALL (▲) (A), and on malignant T cells of 14 patients with T-ALL or T-LL (B). Results are expressed as ID50 of IT in the presence of 6 mM NH_4Cl in relation to the maximum binding of ^{125}I-WT1 per cell. MoAb binding and ID50 were both the mean of triplicates of three independent experiments. Standard deviation was less than 10%. Standard line was obtained with linear regression analysis.

Protein synthesis inhibition of malignant T cells

Protein synthesis inhibition of WT1-ricin A in the presence of 6 mM NH_4Cl in malignant T cells of patients with T-ALL or T-LL was calculated from the dose response curves and expressed as ID50 (Table I). Malignant cells from non-T origin from patients with C-ALL or B-ALL were used as controls. They showed no CD7 expression and hardly any inhibition of protein synthesis (ID50 $>10^{-7}$ M). Malignant T cells were variably sensitive to WT1-ricin A. As shown in Table I, the cells from different patients varied considerably in antigen density. Comparison of the ID50-values and the number of CD7 binding sites per cell by linear regression

Table I. Experimental data of patients.

Patient	Diagnosis	Ag-density[1] (Mol./cellx10^{-3})	ID50[2] (M)
1.	T-ALL	21	10^{-9}
2.	T-ALL	45	$4x10^{-10}$
3.	T-LL	39	$3x10^{-10}$
4.	T-ALL	85	10^{-11}
5.	T-LL	74	$1.5x10^{-11}$
6.	T-ALL	250	10^{-11}
7.	T-LL	35	$1.5x10^{-10}$
8.	T-ALL	87	$7x10^{-12}$
9.	T-LL	30	10^{-10}
10.	T-LL	5	$3x10^{-8}$
11.	T-ALL	29	$2x10^{-10}$
12.	T-ALL	118	$3x10^{-11}$
13.	T-ALL	65	$4x10^{-11}$
14.	T-LL	48	$6x10^{-11}$

[1]Antigen density of CD7 on the malignant cells of patients with T-ALL or T-LL as measured by ^{125}I-WT1 expressed in average number of molecules per cell.

[2]ID50 of WT1-ricin A in the presence of 6 mM NH_4Cl.

Data are the results of at least three experiments.

analysis showed a good correlation for most patients ($p<0.001$; Fig 1B). The regression line was almost similar to the line found for T cell lines. Only the data of patient 6 with the highest antigen density on his malignant cells diverged from the data of the other patients. Statistical analysis of the 95% confidence limits of the antigen density and the ID50 in cell lines and in malignant T cells of patients showed a significant relation, which suggests that the cytotoxicity of WT1-ricin A to cell lines and leukaemic cells is comparable.

Cell kill by WT1-ricin A

The protein synthesis inhibition by WT1-ricin A in malignant T cells is comparable to that observed in T cell lines. Presently no reproducible and satisfactory clonogenic assay of malignant T cells is available to determine the exact amount of cells eliminated by an IT. The killing potency of the IT treatment to malignant T cells is approached by a cloning assay with the human T cell line: CEM, that expresses CD7 antigen in comparable amounts as the malignant T cells of most patients. In this assay an average cloning frequency of untreated cells of 90% in the absence and 65% in the presence of NH_4Cl with an intra-assay standard deviation of less than 10% was achieved. Cells were exposed to various concentrations of WT1-ricin A with or without NH_4Cl and cultured in various cell concentrations to determine the number of residual clonogenic cells after treatment (Fig 2). WT1-ricin A (10^{-8} M) in the absence of NH_4Cl reduced almost 99% (2-logs) of the clonogenic cells, but in the presence of 6 mM NH_4Cl 10^{-9} M IT reduced the number of clonogenic cells with 6-logs (0.0001% residual cells, n=3). A higher IT concentration (10^{-8} M) resulted even in a more substantial kill exceeding 6-logs. The exact degree of elimination at that concentration could not be determined due to the seeding limit (10^6 cells) of the assay. These results suggest that 10^{-8} M WT1-ricin A with 6 mM NH_4Cl may be extremely effective for the elimination of malignant clonogenic T cells from bone marrow.

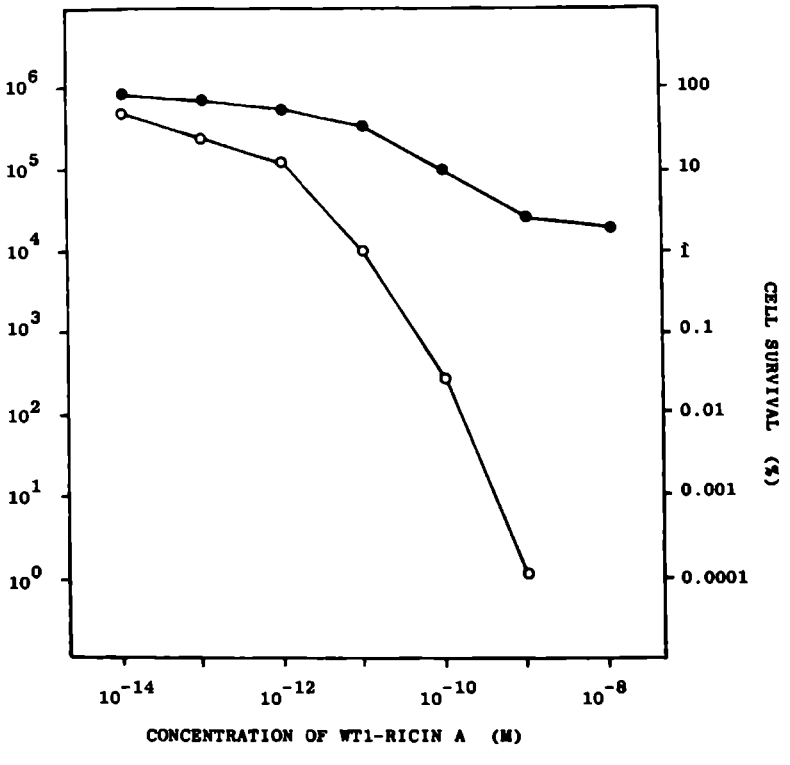

Fig. 2. Cell survival after treatment of CEM with WT1-ricin A with or without NH_4Cl assessed by clonogenic assay. Cells were incubated with various concentrations WT1-ricin A in the absence (●) or the presence (○) of 6 mM NH_4Cl. Number of surviving cells was calculated from the cloning frequency of untreated cells. Each point represents the mean of triplicate cultures from three experiments. Standard deviations were less than 10%.

Internalization of WT1

Cytotoxicity of IT depends on the amount internalized. In order to decrease the possibility of haematopoietic stem cell reduction and to optimalize the elimination of malignant cells from the bone marrow, we studied the influence of concentration and incubation time on the internalization of the IT. To determine the influence of the concentration on the amount of cell bound and internalized MoAb CEM

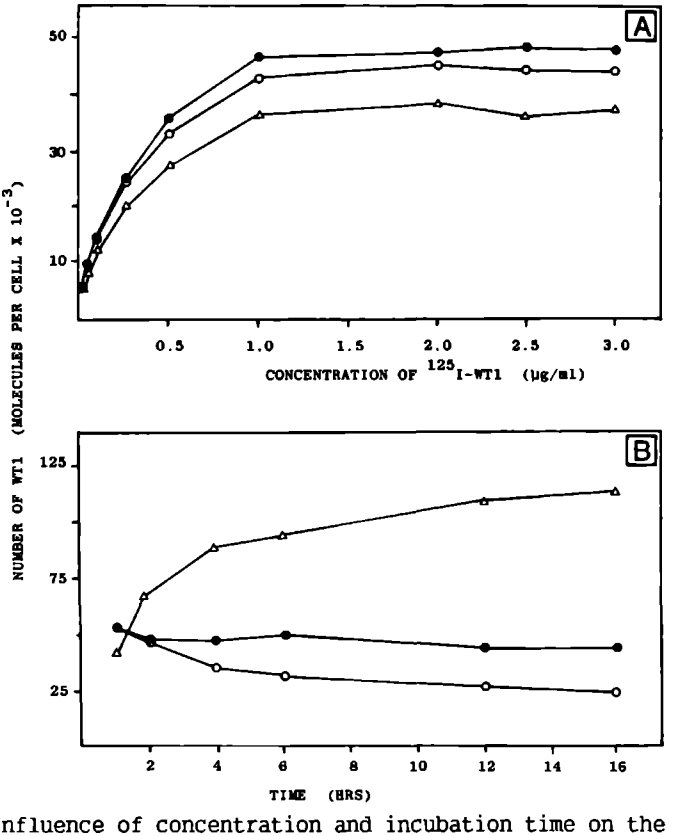

Fig. 3. Influence of concentration and incubation time on the binding at at 4°C (●) and 37°C (○), and internalization (△) of WT1 into CEM. CEM cells were incubated for 1 hr with ^{125}I-labelled WT1 in various concentrations (A), or in fixed concentration (B, 2 µg/ml) for various periods at 4°C and 37°C.

cells were incubated with various concentrations of ^{125}Iodine-labelled WT1 at 4°C and 37°C for 1 hr, followed by a stripping procedure to remove surface bound antibody (Fig 3A). Both incubations at 4°C and at 37°C with a MoAb concentration higher than 1 µg/ml (6.7×10^{-9}M) resulted in a saturation of the cell binding potency. When CEM was incubated at 37°C, a slightly lower antigen expression was found. The amount of internalized MoAb was directly proportional to the amount of cell bound MoAb resulting in a percentage of internalization (76%) which remained equal over a wide range of concentrations.

The kinetics of WT1 internalization was determined with a saturating concentration of ^{125}I-WT1 (2 µg/ml; Fig 3B). The amount of MoAb bound to CEM at 4°C was not significantly changed in time. The amount of surface bound MoAb at 37°C decreased in time, but CD7 expression never disappeared completely. WT1 was rapidly internalized during the first 4 hours. Thereafter the internalization slowed down, but WT1 continued to accumulate intracellularly even after 16 hrs. The number of internalized WT1 molecules surpassed the number of cell bound WT1 within 1.5 hr suggesting antigen reappearance after internalization.

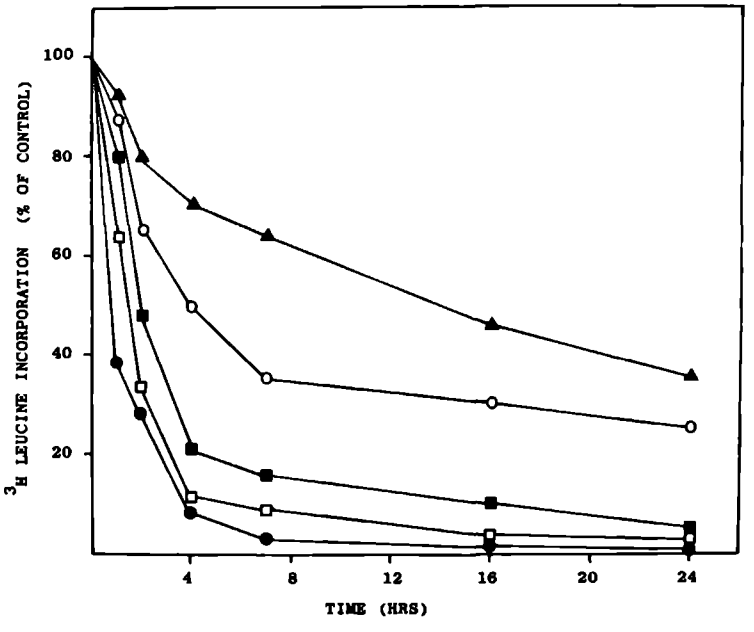

Fig. 4. Cytotoxicity kinetics of WT1-ricin A in the presence of ammonium chloride to T cell lines. Cells were incubated with 10^{-8} M WT1-ricin A in the presence of 6 mM NH_4Cl for various periods of time followed by an 1 hr uptake of ^{3}H-leucine. Inhibition of protein synthesis is expressed as percentage ^{3}H-leucine incorporation of untreated controls. Each point represents the mean of triplicate incubation from three experiments. Standard deviations were less than 10%. (●) GH1; (□) Jurkat; (■) CEM; (○) HSB-2; (▲) HPB-ALL.

The internalization kinetics determined by this assay was limited to 16 hrs since after that time a pronounced cell mortality occurred due to irradiation. Incubation for 24 hrs with a nonsense ^{125}I-MoAb resulted in a 80% cell loss as determined by trypane blue exclusion, whereas unlabelled WT1 did not influence the viability.

With a nonsaturating concentration (0.2 µg/ml WT1) cells required a longer period to attain the same amount of internalized antibody as cells incubated with a saturing concentration (data not shown).

Kinetics of protein synthesis inhibition

In order to study the influence of the incubation time directly on the cytotoxicity of WT1-ricin A the kinetics of protein synthesis inhibition was determined. The relation between the cytotoxic efficacy of WT1-ricin A and the antigen density was also observed during kinetic studies with 10^{-8} M (1,5 µg/ml) IT in incubation periods up to 24 hrs (Fig 4). High antigen density on the target cells resulted in a fast inhibition of the protein synthesis. Inhibition of 50% of the ^{3}H-leucine incorporation in GH1 was achieved within 1 hr in contrast to 14 hrs for HPB-ALL. The inhibition of protein synthesis in time was comparable to the pattern of a continuous uptake of IT (Fig 3).

DISCUSSION

Our and other studies have shown that IT offer a promising approach for the elimination of leukaemic cells (Myers et al, 1984; Casellas et al, 1985; Preijers et al, 1988a). Most studies have been carried out on cell lines only, though there may be differences in sensitivity of malignant cells for the IT in comparison to cell lines. Therefore we studied the cytotoxic efficacy of WT1-ricin A on malignant T cells collected from patients before initiation of chemotherapy and compared the data with the cytotoxicity to T cell lines. As recently described (Myers et al, 1984; Preijers et al, 1988a), WT1-ricin A in the presence of ammonium chloride inhibits the protein synthesis of cell lines very effectively depending on antigen expression and exposition

duration of the target cells. We found that the inhibition by WT1-ricin A in malignant T cells was also related to the antigen density, comparable with T cell lines (Preijers et al, 1988a). This implicates, as also suggested by Laurent et al (1986), that the sensitivity of the malignant T cells for the IT differs from one patient to another. The measurement of antigen density on the malignant cells of each patient may predict the cytotoxicity of the IT. The present data suggest that cell lines can be used as a model to screen initially the suitability and optimal killing conditions of the IT. Before clinical application cytotoxicity to the malignant T cells of the individual patients has to be determined to select patients for bone marrow purging.

Many investigators have tried to culture malignant T cells to show the absolute amount of cell kill (Smith et al, 1986; Georgoulias et al, 1986). At present there is no satisfactory cloning method for leukaemic T cells. To approach the clinical situation a clonogenic assay for T cell lines was used. Formally this assay will not be an evidence for leukaemic cell kill. However, data from other clonogenic assays, carried out in liquid culture on all cell lines used in this study, showed that the relation between antigen density and killing capacity of WT1-ricin A was similar to that found with protein synthesis inhibition (to be published).

In the clonogenic assay decribed in this paper an elimination of more than 6-logs of CEM cells was observed with WT1-ricin A in the presence of 6 mM ammonium chloride. These results are comparable to data of Casellas et al (1985) concerning the cytotoxicity of T101-ricin A to CEM. Myers et al (1984) found that after a 3 hrs treatment of GH1 with WT1-ricin A less than 0.1% of the cells survived. These data suggest that ricin A IT are very potent for the elimination of malignant T cells and provide an alternative superior to other reported cytotoxic agents which possess either lower cytotoxicity (complement-mediated kill: Bast et al, 1983; De Fabritiis et al, 1985; Favrot et al, 1986; Preijers et al, 1988c) or which are less specific (Rowley et al, 1987).

In most experiments cell lines and malignant T cells were incubated with IT for 24 hrs. For practical reasons a reduction of the incubation time prior to ABMT is advantageous. The cytotoxicity of IT, however, depends on the internalization capacity of the antigen. Therefore we studied the kinetics of internalization and it appeared that WT1 was

rapidly internalized. Within 1.5 hr the amount of internalized WT1 exceeded the amount of cell bound WT1. This implicates a continuous synthesis or reappearance of antigen on the cell surface. In contrast to data of Myers et al (1984), we found that CD7 did not disappear completely from the cell membrane within 16 hrs explaining the continuous uptake of WT1. The amount of internalized WT1 was strongly related to the amount of cell bound antibody within a wide range of concentrations. This may be important for the use of WT1-ricin A in a cocktail of IT in which each IT has to be diluted to achieve an optimal concentration of ricin A-chain that will cause no nonspecific cytotoxicity.

The continuous internalization of WT1 was reflected in the kinetics of cytotoxicity of WT1-ricin A. The cells were incubated with a saturating IT concentration of 1.5 µg/ml (10^{-8} M). After the initially rapid inhibition within 4 hrs, protein synthesis continued to decrease. These data justify a long incubation time for an optimal elimination of malignant T cells from bone marrow intended for reinfusion. By the continuous internalization of IT also cells with a low density of CD7 have a higher chance to be killed by surpassing the threshold of the amount of internalized ricin A necessary for its deleterious action (Casellas et al, 1982).

As previously observed, bone marrow precursor cells (CFU-GM and BFU-E) are not detectably affected during the long term incubation period (Preijers et al, 1988b). At present 11 patients with T lymphoblastic malignancies have received an autotransplant after incubation of the marrow with 10^{-8} M WT1-ricin A in the presence of 6 mM NH_4Cl for 16 hrs. Two patients who had the lowest antigen (CD7) density on their malignant cells relapsed suggesting an incomplete elimination of malignant T cells from their bone marrow. Two patients died by transplantation related complications. Seven patients are alive and well (median survival 21 months). Extension of autotransplants and a longer follow-up of the patients are required to confirm whether in this much more complicated situation still a relation exists between antigen density and cell kill with relapse-free survival. Clinical details from the ABMT results will be published elsewhere.

From our studies we conclude: T cell lines seem to be useful as model for testing the potency of ricin A-chain IT towards malignant T

cells and to study more basically the way of action. WT1-ricin A is a very potent IT for the purging of bone marrow of patients suffering from T cell malignancies because this IT is highly effective at the saturating concentration of 10^{-8} M. The long incubation period with this IT is justified due to re-expression of the CD7 antigen on the cell membrane. No adverse effects on haematopoietic progenitor cells occur under these conditions.

ACKNOWLEDGEMENT

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CHAPTER 6

AUTOLOGOUS TRANSPLANTATION OF BONE MARROW PURGED IN VITRO WITH ANTI-CD7-(WT1) RICIN A IMMUNOTOXIN IN T CELL LYMPHOBLASTIC LEUKEMIA AND LYMPHOMA.

F.W.M.B. Preijers, T. de Witte, J.M.C. Wessels, G.C. de Gast*, E. van Leeuwen**, P.J.A. Capel*** and C. Haanen.

From Department of Hematology, University Hospital Nijmegen;
*Department of Hematology, University Hospital Utrecht;
**Children's Hospital "Emma Kinderziekenhuis", Amsterdam; and
***Department of Experimental Immunology, State University Utrecht, The Netherlands.

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ABSTRACT

Seven patients with high risk acute T cell lymphoblastic leukemia (T-ALL) and five with T cell lymphoma (T-LL) were treated with autologous bone marrow transplantation (ABMT) after in vitro purging of their bone marrow with WT1 (CD7)-ricin A-chain immunotoxin. CD7 expression on the tumor cells showed large variations between the individual patients and was highly related to the specific cytotoxicity of WT1-ricin A. Incubation of bone marrow with up to 10^{-8} M WT1-ricin A in the presence of 6 mM NH_4Cl did not compromise the growth potential of the hematopoietic progenitors CFU-GM, CFU-GEMM and BFU-E. Hematologic engraftment ($>10^9$ leukocytes/l) occurred within a normal time period (median: 15.5 days). Six patients are alive and in complete remission (CR) at 44+, 40+, 36+, 22+, 7+ and 3+ months after ABMT. Four patients relapsed within 6 months after ABMT. Two of them had the lowest CD7 expression on their tumor cells, the other two were transplanted in CR2 and CR3. Two patients died from transplantation related infections.

The immunologic reconstitution was delayed, although the numbers of T cells reached normal levels within 1 month. The number of $CD7^+$ cells remained low up to one year after transplantation. The T4/T8-ratio was decreased for at least 6 months. The T cell response to mitogens recovered to normal levels after one year. This study shows that ABMT with WT1-ricin A purged bone marrow in high risk T cell malignancies results in a complete hematopoietic and a delayed immunologic reconstitution. The actuarial relapse free survival is 56% at 3 years.

INTRODUCTION

Autologous bone marrow transplantation (ABMT) is increasingly applied as alternative treatment for patients with acute T cell lymphoblastic leukemia (T-ALL) or lymphoma (T-LL) who lack a HLA-compatible donor (1-6). Bone marrow, even when aspirated during complete remission, is probably contaminated with residual malignant T cells, and these may be responsible for relapse after ABMT. Various techniques have been developed to eliminate in vitro the malignant cells from the graft (7-14). Purging of the bone marrow with ricin A-chain immunotoxins (ITs) specifically directed against the malignant T cells appeared to be a promising approach to improve the therapeutical efficacy of ABMT (7-10). Recently we showed that CD7 is a suitable antigen as target for ITs (7,15) since CD7 is expressed in most T cell malignancies. The IT WT1-ricin A (anti-CD7) appeared to eliminate very effectively malignant T cells from the bone marrow, in part due to continuous internalization of the antigen-IT complex by the cells, provided that the CD7 density on the cell surface was high. We demonstrated that WT1-ricin A in the presence of 6 mM ammonium chloride induces more than 6-logs kill of the malignant T cell line 'CEM' (15).

In this paper we report clinical and experimental data of ABMT, using bone marrow purged in vitro with WT1-ricin A, in 12 patients suffering from bad risk T-ALL and T-LL in complete remission.

MATERIALS, METHODS AND PATIENTS

Immunotoxin

Ricin A was kindly provided by Dr F.K. Jansen (Centre de Recherches Clin Midy, Montpellier, France). The murine anti-human T cell MoAb: WT1, anti-CD7 (kindly provided by Dr W.Tax; 16) was conjugated to ricin A by means of succinimidyl 3-(2-pyridyldithio) propionate (SPDP; Pharmacia) as described previously (7).

Antigen denstity

WT1 was labelled with ^{125}Iodine (Amersham International, Amersham, Bucks., UK) using the chloramine T method (37). The concentration and

specific activity of the ^{125}I-labelled MoAb were determined by respectively radioimmunoassay and binding assay with limiting quantities of labelled antibody. The absolute number of binding sites per cell was determined as described previously (7).

Cytotoxicity of WT1-ricin A

Fresh or thawed cryopreserved leukemic cells were incubated in EMEM without leucine (Flow Labs, Irvine, Scotland, UK) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate and gentamycin (50 µg/ml). Each well of U-bottomed plates (Costar, Cambridge, Mass., USA) was filled with 10^5 cells supplemented with varying concentrations of IT in the absence or presence of 6 mM NH_4Cl to a final volume of 200 µl. Cells were incubated for 24 hrs at 37°C in a humidified incubator with 5% CO2 in air, followed by 24 hrs incubation with 0.5 µCi ^{3}H-leucine (TRK510, Amersham). Cells were harvested and radioactivity was counted. The cytotoxicity was expressed as percentage inhibition of ^{3}H-leucine incorporation of untreated cells corrected for the background value determined in the presence of 1 mM cycloheximide.

Cytotoxicity to bone marrow progenitor cells

Bone marrow was aspirated from patients undergoing cardiac surgery after informed consent was obtained and anticoagulated with acid citrate dextrose (ACD). Erythrocytes and mature granulocytes were removed by gradient centrifugation on Ficoll (1.077 g/ml). Cells (10^6/ml) were incubated with ITs (10^{-8} M) with or without 6 mM ammonium chloride for 24 hrs. Cells were washed and the decrease of bone marrow progenitors was determined in clonogenic assays of granulocyte/macrophage and granulocyte/erythrocyte/macrophage/megakaryocyte colony-forming cells (CFU-GM and CFU-GEMM respectively), and erythroid burst-forming cells (BFU-E) as described previously (18).

PATIENTS

ABMT was performed in 12 patients with bad risk T cell malignancies: 7 T-ALL and 5 T-LL. Before ABMT eight patients were in CR1, one in CR2 and three in CR3 (Table 1). None of these patients had an HLA-identical sibling donor. The mean age was 21 years (range 10-35 years). Data of phenotype of the malignant T cells and clinical status of the individual

Table 1. Clinical data of patients before ABMT.

Pat. No.	Age/ Sex	Initial Diagnosis/ Stage	Data At Diagnosis WBC 10^9/l	LDH U/l	Hep.	Spl.	Status Before ABMT	Phenotype Of Malignant cells
1.	27/M	T-ALL	30	330	+	+/-	CR1	CD1, CD2, CD3, CD4, CD5, CD7
2.	17/M	T-ALL	56	1487	-	+	CR1	CD7
3.	35/M	T-ALL	204	9700	+	+	CR1	CD1, CD7
4.	24/M	T-ALL	93	305	+	-	CR1 CNS	CD7
5.	11/M	T-ALL	29	620	+	+	CR2 CNS	CD2, CD4, CD5, CD7
6.	22/M	T-ALL	33	1483	-	+	CR1	CD2, CD5, CD7, CD8
7.	10/F	T-ALL	333	2238	+	+	CR3 CNS	CD1, CD5, CD7, CD8
8.	26/F	T-LL, 3A	9	201	-	+	CR1	CD7
9.	15/M	T-LL, 4A	165	4000	+	+	CR3	CD7
10.	17/M	T-LL, 4A	3	193	-	-	CR3 CNS	CD7
11.	31/M	T-LL, 4A	4	292	-	+	CR1	CD7
12.	28/M	T-LL, 3A	6	410	-	-	CR1 CNS	CD2, CD5, CD7

ABMT, autologous bone marrow transplantation; CNS, previous central nervous system localization; CR, complete remission followed by remission phase; Hep, hepatomegaly; LDH, lactic dehydrogenase in Units/l; Spl. splenomegaly; T-ALL, acute T-cell lymphoblastic leukemia; T-LL, T-cell lymphoblastic lymphoma followed by staging; WBC, white blood cell counts.

patients at time of diagnosis and before ABMT are given in Table 1. Antigen density and cytotoxicity of WT1-ricin A to the malignant cells was determined prior to the marrow harvest.

Bone marrow processing

Bone marrow was aspirated from the posterior iliac crests under general anesthesia and anticoagulated with heparine. Hematopoietic progenitor cells were enriched by density floatation separation in Percoll gradients (17). The low density fraction ($d \leq 1.070$ g/ml) of the separated bone marrow was incubated with 10^{-8} M WT1-ricin A in the presence of 6 mM ammonium chloride in RPMI 1640 supplemented with 5% FCS, 2 mM glutamine and 50 µg/ml gentamycin at 37°C for 16 hrs. After incubation cells were washed, cryopreserved and stored until the day of reinfusion (designated day 0) (19). Before reinfusion the recovery of hematopoietic progenitors CFU-GM and BFU-E before and after IT treatment and after cryopreservation was determined (18) to check the procedure. Before and after all steps cell recovery and morphology were assessed in each fraction.

Transplantation procedure

Patients were conditioned with cyclophosphamide (CY) 60 mg/kg under protection of 12 mg/kg 2-mercaptopurinethanesulfonate (Mesna) to prevent hemorrhagic cystitis on days -6 and -5 and total body irradiation (TBI) with a total dose of 800 cGy given in a single fraction or HD-melfalan 180 mg/m² (patient 5) on day -1. All patients received central nervous system prophylaxis with intrathecally methotrexate. On day 0 purged bone marrow was thawed rapidly and reinfused immediately. Daily blood counts were performed during hypoplasia to define the day of engraftment (leukocyte count $>1 \times 10^9$/l). Platelet levels were maintained above 20×10^9/l with irradiated (20 Gy) platelet transfusions.

Follow up of T cell recovery

Heparinized venous blood and bone marrow aspirates (sternum) were obtained from every patient 1, 3, 6 and 12 months posttransplantation. Hematologic reconstitution and cell numbers were determined in blood count analysis and morphology of blood and bone marrow smears.

The reconstitution of lymphocyte subpopulations was determined by

flow cytometry of the lymphocyte scatter area and indirect immunofluorescence of bound monoclonal antibodies (MoAbs). The following anti-T cell MoAbs were used: WT32 (CD3; 20), WT1 (CD7; 16) and WT82 (CD8; 20) provided by Dr W. Tax; OKT6 (CD1; 21), OKT4 (CD4; 21), OKT1 (CD5; 21) from ORTHO Diagnostic Systems Inc (Raritan, NJ). Additionally were used: OKB2, identifying B-cells pre-B cells and granulocytes (22), OKB7 (identifying B cells expressing the 175 kD antigen; 22); OKM1 (CD11; 23) and OKM5 (recognizing monocytes and platelets expressing the 88 kD antigen; 24). After the initial incubation with the concerning MoAbs the cells were incubated with FITC-labelled Goat-anti-mouse-IgG $f(ab')_2$-fragment (GAM-FITC; American Qualex International Inc., La Mirada, California).

The functional reconstitution of the T lymphocytes was determined in stimulation assays carried out in 96-wells U-bottom plates. Each well was filled with 10^5 cells in culture medium in the absence or the presence of 40 µg/ml phytohaemagglutinin (PHA) or 15 µg/ml concanavalin A (Con A) to a final volume of 200 µl and incubated at 37°C in a humidified atmosphere with 5% CO2 in air for 48 hrs, followed by a labelling with 0.5 µCi ^{3}H-thymidine (TRK 61, Amersham) for 24 hrs. Thereafter cells were harvested and radioactivity was counted. The PHA or Con A stimulation of lymphocytes of the patients was expressed as percentage ^{3}H-thymidine incorporation by PHA or Con A stimulated lymphocytes of normal individuals, all corrected for background incorporation of unstimulated cells.

Statistical analysis

Differences in leukocyte number and hematopoietic progenitors before and after IT treatment were statistically analyzed by the matched-pair t-test.

To study the adverse effect of IT to the bone marrow the hematopoietic recovery was examined by the Kaplan-Meier estimate of probability (25). The day of engraftment was defined by the day after reinfusion with a leukocyte count of $>1.0 \times 10^9$/l and rising counts thereafter. After ABMT patients with no evidence of relapse were classified as being in complete remission. The overall survival and the probability of relapse posttransplant were evaluated by the Kaplan-Meier method.

Table 2. In vitro data of patients.

Patient No.	Ag-density[1] (Mol./cellx10^{-3})	ID50[2] (M)
1.	21	10^{-9}
2.	45	$4x10^{-10}$
3.	85	10^{-11}
4.	9	ND*
5.	85	$4x10^{-11}$
6.	29	$2x10^{-10}$
7.	118	$3x10^{-11}$
8.	39	$3x10^{-10}$
9.	74	$1.5x10^{-11}$
10.	250	10^{-11}
11.	87	$7x10^{-12}$
12.	30	10^{-10}

[1]Antigen density of CD7 on the malignant cells of the patients as measured by ^{125}I-WT1 expressed in average number of molecules per cell. [2]ID50 of WT1-ricin A in the presence of 6 mM NH_4Cl.
*ND = not determined.

RESULTS

Cytotoxicity of WT1-ricin A to malignant T cells in vitro

The cytotoxic potency of WT1-ricin A in the presence of 6 mM NH_4Cl was tested on the malignant cells of eleven out of twelve patients and expressed as dose required for 50% reduction of the protein synthesis (ID50). Malignant cells before initial therapy of patient 4 were not available for cytotoxicity studies. Protein synthesis was inhibited mainly depending on antigen density of CD7 on the cell surface (Table 2). The average antigen density varied individually from 9000 to 250,000 molecules per cell. As described previously, a strong correlation was

found between cytotoxicity and antigen density of CD7 (15). Malignant cells from patients with C-ALL and B-ALL without CD7 expression were used as controls.

Effect of WT1-ricin A on hematopoietic progenitors

The cytotoxicity of WT1-ricin A with or without NH_4Cl to hematopoietic progenitors was determined by means of clonogenic assays. Cells were incubated with 10^{-8} M WT1-ricin A, the dose used to treat large amounts of bone marrow. In comparison with untreated cells no influence of IT treatment in the presence of 6 mM NH_4Cl was found on the plating efficiency of CFU-GM (97 $\pm$ 11%), CFU-GEMM (101 $\pm$ 11%) and BFU-E (94 $\pm$ 10%) as determined in 5 independent experiments.

Purging of bone marrow

Low density marrow cells (d<1.070 g/ml) were incubated with 10^{-8} M WT1-ricin A in the presence of 6 mM NH_4Cl. This resulted in a slight reduction of nucleated cells (mean reduction 10%), CFU-GM and BFU-E (Table 3), that was probably not caused by cytotoxicity of the IT but largely due to the procedure itself. Patients received a mean cell dose per kg of 7.5 $\pm$ 2.7 x 10^7 (range 4.5 to 13.4 x 10^7) nucleated cells, 11.2 $\pm$ 9.5 x 10^4 (range 1.9 to 37.6 x 10^4) CFU-GM and 17.2 $\pm$ 11.7 x 10^4 (range 3.7 to 42.9 x 10^4) BFU-E.

Hematologic engraftment

All patients engrafted after reinfusion of WT1-ricin A purged bone marrow. The mean day of recovery of leukocyte count to >10^9/l was day 17.1 $\pm$ 5.1 (median: 15.5; range 9 to 25 days) (Fig 1, Table 4). After recovery of leukocytes to >1.0x10^9/l patient 6 was treated with ganciclovir, fluconazole and flucytosine because of the suspicion of CMV infection and a proven Candidemia. Subsequently leukocyte count dropped and the patient died in aplasia. Platelet recovery (>50x10^9/l), was delayed in 3 patients (patients 8, 10 and 11) and was not evaluable in 2 (patients 6 and 9). The median recovery occurred on day 38 (range day 14 through 270). No relation was found between the state of disease, neither the number of reinfused hematopoietic progenitor cells (CFU-GM and BFU-E) nor the day of engraftment.

Table 3. Recovery after in vitro bone marrow purging.

Patient No.	WBC		CFU-GM		BFU-E	
	Before IT ($x10^{-9}$)	After IT ($x10^{-9}$)	Before IT ($x10^{-6}$)	After IT ($x10^{-6}$)	Before IT ($x10^{-6}$)	After IT ($x10^{-6}$)
1.	3.65	3.12	9.5	4.8	6.7	4.5
2.	6.64	5.60	15.0	12.5	24.8	23.5
3.	4.01	3.60	4.6	3.2	12.0	9.6
4.	9.80	9.00	24.2	14.7	28.6	14.7
5.	2.90	2.80	3.5	3.5	9.4	9.1
6.	3.90	3.30	2.2	1.2	2.3	2.3
7.	2.80	2.20	2.3	2.4	5.3	4.2
8.	5.65	5.65	31.4	24.1	39.0	27.5
9.	4.85	3.91	4.6	4.6	15.6	8.4
10.	6.54	5.76	3.6	2.3	8.8	5.1
11.	4.60	4.50	3.6	3.2	6.7	6.7
12.	9.80	9.40	6.4	4.6	6.4	6.4
X±SD	5.43±2.39	4.90±2.32	9.2±9.5	6.8±6.8	13.8±11.2	10.2±7.9
Range	2.8-9.8	2.2-9.4	2.2-31.4	1.2-24.1	2.3-39.0	2.3-27.5
	¦-P<0.0025-¦*		¦-P<0.025--¦		¦-P<0.0125-¦	

Evaluation of recovery after incubation of the bone marrow from patients with WT1-ricin A and NH_4Cl. WBC, white blood cells.

*Correlation determined by the matched pair t-test.

Table 4. Clinical data after ABMT.

Patient No.	Diagnosis Before ABMT	Engraftment* WBC (days)	Engraftment* PLT (days)	Status After ABMT
1.	T-ALL; CR1	14	22	Died after 7 mths in relapse (6 mths post-ABMT).
2.	T-ALL; CR1	17	32	Alive and CR, >44 mths.
3.	T-ALL; CR1	14	38	Alive and CR, >36 mths.
4.	T-ALL; CR1	9	14	Died after 7 mths in relapse (6 mths post-ABMT).
5.	T-ALL; CR2	21	38	Alive and relapse (5 mths post-ABMT), >11 mths.
6.	T-ALL; CR1	13	NE**	Died after 51 days by Candida and CMV.
7.	T-ALL; CR3	14	40	Alive and relapse (5 mths post-ABMT), >8 mths.
8.	T-LL 3A; CR1	13	180	Alive and CR, >40 mths.
9.	T-LL 4A; CR3	20	NE	Died after 61 days by Strept. viridans and CMV.
10.	T-LL 4A; CR3	25	270	Alive and CR, >22 mths.
11.	T-LL 4A; CR1	21	>210	Alive and CR, >7 mths.
12.	T-LL 3A; CR1	25	60	Alive and CR, >3 mths.

ABMT, autologous bone marrow transplantation; CR, complete remission; T-ALL, acute T-cell lymphoblastic leukemia; T-LL, T-cell lymphoblastic lymphoma.

*Recovery of leukocyte (WBC) counts to $>10^9/l$ and platelet counts to $>50x10^9/l$ in days after ABMT.

**Not evaluable.

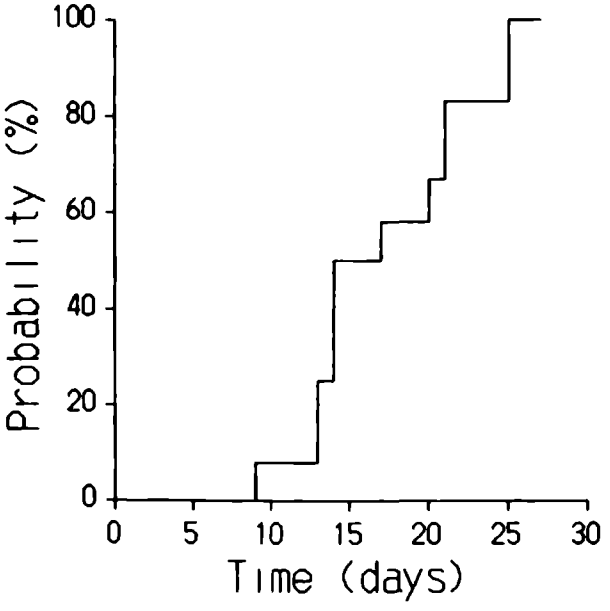

Fig. 1. Recovery of leukocytes to $>10^9/l$ after autologous bone marrow transplantation.

Survival

Clinical data of survival after ABMT are shown in Table 4. Eight of the 12 patients treated for T-ALL and T-LL are alive; two of them (patients 5 and 7) relapsed within 5 months after ABMT in CR2 and CR3. Six patients are still in CR (3+, 7+, 22+, 36+, 40+, 44+ months). Four patients have died; 2 patients died 1 month after recurrence of leukemia, 1 patient died by a Candida and CMV infection (patient 6), and 1 patient by Streptococcus viridans and CMV infection (patient 9).

The calculated overall survival at 3 years is 64.8% and the probability of relapse free survival is 55.6% (Fig 2). The four relapses were found in patients treated for T-ALL: 2 of these patients (1 and 4) had a low CD7 expression on their malignant cells. The other two patients were transplanted in CR2 and CR3 respectively. In the group of T-LL patients 4 of the 5 patients are alive and disease free 3, 7, 22 and 40 months after ABMT.

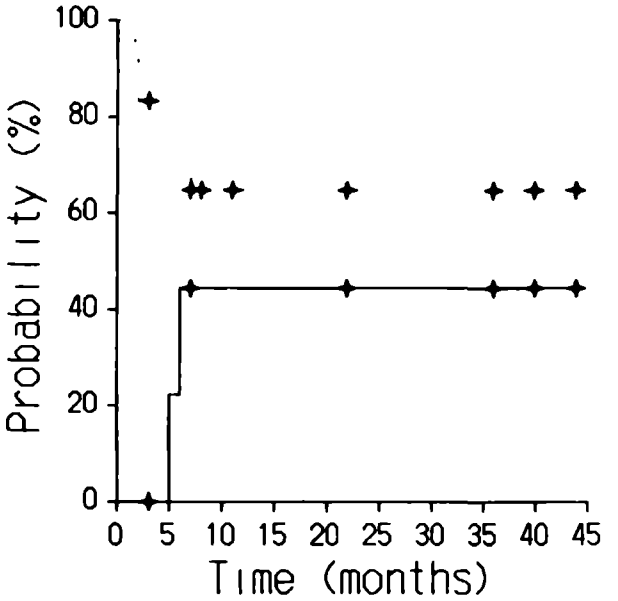

Fig. 2. Kaplan-Meier estimate of the probability of actuarial relapse rate (——) and overall survival (...) of 12 patients with T-ALL or T-LL after autologous transplantation with WT1-ricin A purged bone marrow. Vertical marks represent surviving patients (|).

Immunologic recovery

The patients who survived more than 1 year were followed for their hematologic and immunologic recovery (Table 5). Leukocyte counts normalized more than 6 months after ABMT, partly due to a longlasting granulocytopenia, whereas lymphocytes and monocytes achieved normal levels within 1 month. The immunologic recovery was delayed for at least 1 year as determined by flow cytometric analysis of the lymphocyte phenotypes. $CD3^+$ and $CD5^+$ cells returned to normal levels within 1 months after ABMT. However, the T4/T8-ratio was reversed for more than 6 months and did not return to normal levels for at least 1 year. This decreased T4/T8-ratio was due to a low number of $CD4^+$ cells whereas $CD8^+$ cells returned to normal levels within 1 month. The number of $CD7^+$ cells did not reach normal values for at least 1 year. The functional recovery was tested by responsiveness to mitogens compared to normal individuals (Table 6). T cells hardly responded to mitogens in the first 3 months, but returned to normal within 1 year after ABMT.

Table 5. Hematologic and immunologic recovery in patients who survived more than 1 year after ABMT.

	Months after ABMT			
	1	3	6	12
Leukocyte	1.8±1.0	2.5±1.3	3.8±1.4	4.8±1.4
Lymphocyte	0.73±0.30	0.96±0.56	0.95±0.57	1.37±0.68
	(42±11)*	(37±8)	(26±12)	(27±15)
Monocyte	0.23±0.20	0.23±0.14	0.33±0.11	0.49±0.32
	(12±6)	(10±5)	(9±3)	(9±6)
Granulocyte	0.76±0.50	1.31±0.80	2.40±0.98	2.89±0.87
	(42±14)	(47±8)	(64±11)	(62±19)
CD1	0.02±0.01	<0.01	<0.01	<0.01
	(3±2)	(<1)	(<1)	(<1)
CD3	0.68±0.35	0.52±0.30	0.64±0.38	0.88±0.50
	(78±9)	(76±11)	(85±9)	(75±6)
CD4	0.28±0.09	0.18±0.05	0.21±0.04	0.45±0.23
	(37±10)	(28±14)	(32±19)	(41±10)
CD5	0.52±0.29	0.41±0.25	0.64±0.38	0.85±0.50
	(63±18)	(59±14)	(74±6)	(73±7)
CD7	0.12±0.01	0.23±0.12	0.29±0.19	0.67±0.70
	(18±10)	(33±2)	(48±13)	(47±29)
CD8	0.43±0.28	0.35±0.21	0.34±0.21	0.42±0.25
	(47±11)	(50±10)	(39±3)	(37±6)
anti-B cell	0.08±0.06	0.05±0.03	0.09±0.06	0.15±0.08
	(9±9)	(8±2)	(7±5)	(9±6)

Values are expressed as mean number of cells $x10^{-9}$ ± standard deviation.
*Percentage of cells between parentheses.

Table 6. Functional recovery of lymphocytes in patients after ABMT.

	Months after ABMT			
	1	3	6	12
PHA	3±3	14±2	42±10	101±11
Con A	2±3	8±6	41±27	97±9

Values are expressed as mean percentage of ^{3}H-thymidine incorporation by cells of normal individuals ± standard deviation of 5 measurements. Average incorporation by lymphocytes of five normal individuals was 37,104 ± 1441 cpm after PHA stimulation and 26,691 ± 2608 cpm after Con A stimulation.

DISCUSSION

The first clinical results of ABMT after in vitro bone marrow purging with the anti-T cell IT, WT1-ricin A, are promising. All patients, 7 with T-ALL and 5 with T-LL, had a bad prognosis at time of diagnosis (36). Although MoAbs with complement lysis are most commonly used for purging, ITs appear often more efficient in cell killing (28,29). Previously we demonstrated that WT1-ricin A in the presence of 6 mM ammonium chloride induces more than 6-logs kill of the malignant T cell line 'CEM' (15). CEM cells express intermediate amounts of CD7 comparable to the CD7 density on malignant T cells of most patients. In vitro experiments showed that the cytotoxic efficacy of WT1-ricin A is highly related to the antigen density of CD7 on the malignant cell surface, which may imply effective reduction of the residual leukemic cells in the bone marrow of most patients.

A prolonged purging procedure of bone marrow with IT may be harmful to the hematopoietic stem cells. We have chosen for an intermediate incubation period of 16 hrs based on kinetics of cytotoxicity and internalization of cell bound IT (15). In our studies we showed that WT1-ricin A is continuously internalized in target cells coinciding with increasing cytotoxicity. In this and in other studies (26), no cytotoxicity of IT to hematopoietic precursors was found after 24 hrs incubation. This contrasts with the effects of cytostatic drugs when used in bone marrow purging (14,27). Bone marrow purging with ITs resulted in a slight reduction of hematopoietic precursors probably caused by intensive manipulation of the marrow.

All patients in this study showed a fast engraftment of the bone marrow as monitored by the leukocyte counts $>10^9/l$ (median day 15.5). The marrow recoveries were rapid compared to previous reports of nonpurged and otherwise purged autologous bone marrow (4,5,9,14,30,31), and comparable with the results of syngeneic marrow engraftment (32). These results provide evidence that sufficient hematopoietic precursor cells were present in the graft and survived the WT1-ricin A purging. In contrast the platelet recovery to $50x10^9/l$ was strongly delayed in 3 patients. The same phenomenon was observed in other studies (3,5,14,30).

Although the number of lymphocytes ($CD3^+$ and $CD5^+$) attained normal levels within one month the immunologic recovery appeared delayed for almost one year. $CD7^+$ cells remained low for at least 6 months. Data of de Gast et al (39), suggesting that the recovery of T cell subsets is mainly due to the proliferation of mature T cells in the graft, may be an explanation for this observation. No T cells with an immature phenotype (CD1) were detected after 1 month. Functional recovery, as measured with response to mitogens, remained low for almost one year. This may be due to a defective T-helper function as observed by Armitage et al (40) resulting in a decreased T4/T8-ratio. The delayed immunologic recovery appeared not to be induced by the WT1-ricin A treatment because other investigators have found similar results in ABMT with other purging procedures or even without purging (30,40,41) and in allogeneic BMT (42).

From the seven patients with T-ALL in the current study four patients relapsed whereas from the 5 patients with T-LL none relapsed. Whether the recurrence of leukemia was caused by residual leukemic cells

in the patient or in the bone marrow graft cannot be determined. Both mechanisms may play a role since two of the relapsed patients had a low CD7 expression on the malignant cells and otherwise T-ALL with a low CD7 expression are less sensitive to high dose chemoradiotherapy. Determination of the CD7 expression on the malignant cells of patients who relapsed after ABMT showed no change in antigen density or antigen heterogeneity, suggesting that the recurrent leukemic cells were not an antigen negative selected population. Moreover, the cytotoxicity of WT1-ricin A to these cells appeared not to be changed (data not shown). This contrasts with data of Thorpe et al. (33) who showed that surviving cells after IT treatment were resistent to the IT. Antigen density of the malignant cells of the other two relapsed patients, who were transplanted in CR2 and CR3, was high. Residual leukemic cells in these patients seem more likely the cause of relapse. Our results suggest that for an effective purging only leukemias with high antigen density on the cell surface should be selected. This may decrease the high relapse rate reported in most ABMT studies with immunologic purged bone marrow (31,34,35). Besides the state of disease influences the outcome of ABMT.

Statistical evaluation of survival after ABMT is virtually impossible because of the low number of patients and the heterogeneic group of diseases. In the group of patients transplanted for T-ALL (7 patients) the survival was 57% , 43% remained in CR, which is comparable with data of Gorin et al (1). The survival is higher than found by Kersey et al (34). In the group of T-LL none of the patients relapsed, but 1 patient died due to a nonleukemic complication. Although this group is too small for statistical analysis our results seem similar to those of others obtained in lymphoblastic lymphoma (3,4).

The advantage of bone marrow purging is difficult to assess due to the various methods used for marrow purging and the lack of studies with large numbers of patients. Gorin et al (38) demonstrated by analysis of 125 patients with ALL in first CR that, although no statistically significant advantage for purging exists, patients who received purged bone marrow had a higher percentage of disease free survival in 3 years (58%) than patients with nonpurged bone marrow (30%).

We conclude that purging of bone marrow with WT1-ricin A prior to ABMT is a safe therapeutic option. It may be the treatment of choice for patients with high risk T cell malignancies.

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CHAPTER 7

CONCLUDING REMARKS.

1. CONCLUSIONS

Ricin A-chain immunotoxins (ITs) are very effective in eliminating malignant and normal T cells in bone marrow grafts. This is achieved by coupling target specific monoclonal antibodies (MoAbs) to the cytotoxic ricin A-chain. ITs compete with and are often superior to other methods of elimination due to their specific cytotoxicity. However, not all hybrids of MoAb and toxin are suitable as IT. In this thesis the factors which determine the cytotoxicity and the clinical use of ricin A-chain ITs are investigated.

ITs have to be internalized into the target cell in order to exert their cytotoxic action. The number of internalized molecules rather than the antigen density on the cell surface determines the cytotoxicity. Antigens which do not internalize the IT are not suitable as target for ITs. Moreover the intracellular transport of the ricin A is important for the cytotoxic efficacy and varies from one type of cell to another. ITs directed against CD3, CD5 and CD7 are rapidly internalized and have highly cytotoxic efficacy to both malignant and normal T cells. Those directed against CD4 and CD8 are either not internalized or only to a small extent and therefore exert a low cytotoxic effect.

The susceptibility of target T cells appears to be related to the state of activation: the susceptibility of nonproliferating normal T cells is lower than that of T cell lines, while PHA-stimulated T cells are effectively inhibited. The increased susceptibility of stimulated T cells appears to be related to cell activity rather than antigen density.

Lysosomotropic agents such as ammonium chloride strongly enhance the cytotoxicity of ricin A-chain ITs depending on the target antigen. NH_4Cl causes a delay in transport of ricin A from endosome to the lysosome and increases the pH of lysosomes to suboptimal values resulting in a decreased destruction of ricin A molecules by lysosomal enzymes. Ammonium chloride in a concentration of 20 mM greatly enhances the cytotoxicity of unstimulated T cells in vitro. The degree of T-lymphocyte depletion from human bone marrow grafts is sufficient to prevent GVHD after allogeneic transplantation. This suggests that higher intracellular concentrations of ricin A can overcome the lower suscep-

tibility of resting normal T cells.

Although the T cell population can be eliminated by the combination of anti-CD3, anti-CD5 and anti-CD7 MoAbs plus complement, ITs in the presence of 20 mM NH_4Cl appear to be more effective than complement treatment. Extensive T cell depletion of the graft, however, may increase the risk of a delayed reconstitution or even rejection of donor marrow after allogeneic bone marrow transplantation (BMT); besides the graft-versus-leukemia (GVL) effect may be compromised. Therefore elimination of subpopulations without simultaneous loss of the GVL potential may be a more reasonable approach. Selective elimination without loss of NK-activity can be effectively obtained with anti-CD4 and anti-CD8 with complement. The appropriate ITs, however, are ineffective due to the low internalization of CD4 and CD8 antigens. The natural killer (NK) activity is little influenced by MoAbs directed against CD3 and CD5 with complement or as ITs, whereas anti-CD7 MoAbs strongly decrease the NK-activity. Therefore a combination of anti-CD3 and anti-CD5 MoAbs with complement or as ITs is recommended in order to eliminate T cells in marrow grafts.

Malignant T cells are, in contrast to normal resting T cells, very sensitive to ITs and as such can be compared to T cell lines. These can be used to mimic the killing potency of ITs to malignant T cells in bone marrow. At present exact assessment of the elimination of malignant cells is not feasible due to the lack of a suitable and reproducible clonogenic assay.

ITs directed against the CD7 antigen are very suitable for the purging of bone marrow contaminated with acute T lymphoblastic leukemia or lymphoma cells because: a. the appropriate antigen is expressed in high quantities in almost all cases of T-ALL or T-LL, b. the IT is continuously internalized emphazising the importance of prolonged incubation for elimination even of cells with a low antigen density, c. protein synthesis of malignant T cells is strongly inhibited by anti-CD7 IT, and d. the viability of a malignant T cell line with an intermediate CD7 density is reduced by more than 6-logs after incubation with IT.

Bone marrow intended for reinfusion can be safely incubated with ricin A-chain ITs without significant loss of hematopoietic progenitor cells. The engraftment and hematologic reconstitution are not delayed when compared with reinfusion data of nonpurged bone marrow. Although

the results of autologous bone marrow transplantation after purging with anti-CD7 IT are promising at present no definitive conclusion can be drawn about its efficacy in comparison with nonpurged bone marrow due to the limited size of the treated patient group.

2. FUTURE PERSPECTIVES

At present some ITs can be used for the in vitro elimination of malignant cells present in auto-grafts and of normal T cells in donor bone marrow. But as long as complete elimination of contaminating malignant cells cannot be guaranteed, investigations must focus on improving the cytotoxicity of ITs. Variation in toxins or coupling reagents may further improve the cytotoxicity. Moreover, a balanced cocktail of ITs, varying in toxins or MoAbs, may be the solution to the heterogeneous expression of antigens. Biological response modifiers (BRMs), such as interleukin-2 and interferon-gamma, may play an important role by increasing the number of binding sites. BRMs may be used in combination with ITs or administered to the patient in vivo before bone marrow aspiration.

At present 85% of the potential candidates for BMT do not receive a transplant due to the lack of a histocompatible sibling and the increased risk of graft-versus-host disease (GVHD) and its related complications which are more pronounced with increasing age. Therefore BMTs with partly matched family donors or histocompatible unrelated donors in combination with effective T cell depletion, and a more stringent conditioning of the patient will increase the number of patients that may benefit from allogeneic BMT. Although GVHD may be prevented in BMT with highly T cell depleted bone marrow, the risk of graft rejection and relapse increases considerably. Whether GVHD and GVL are mediated by one or different subpopulations of lymphocytes is not known and requires further study.

Apart from normal T cells in allogeneic bone marrow grafts and contaminating malignant cells in autologous grafts, residual cells in the patient that are not killed by sublethal dose of chemoradiotherapy are the main cause of relapse. Anti-cancer drugs have a low differential cytotoxicity to resting malignant cells in comparison with normal cells.

rapidly than normal cells. The in vivo application of ITs will provide a significant advance in the treatment of residual disease due to the less nonspecific cytotoxicity of the IT and the killing potency to malignant cells in a less active stage. When it becomes possible to use ITs effectively, dangerous and expensive therapy like BMT may no longer be necessary. However, higher demands are made upon the quality of the ITs because enhancers of cytotoxicity, such as ammonium chloride, cannot be administered to the patient in sufficient quantities. In vivo use of ITs is hampered by instability of the MoAb-toxin complex and rapid clearance by the monocytic-phagocytotic system (MPS). The instability has been overcome with the modification of the coupling reagents, whereas the rapid clearance of ITs by the MPS can be largely overcome by deglycosylation of the mannose and fucose residues on the toxin. These induce a more effective anti-tumour agent because it is cleared from the blood more slowly and so has greater opportunity to localize within the tumor.

On the other hand when in vivo application of ITs becomes feasible this may result in stronger immunosuppression by eliminating radiochemotherapy-resistant normal T cells in the patient. Thus the risk of allogeneic graft rejection due to remaining T cells in the patient after the conditioning treatment may diminish.

Even solid tumors with a low blood-tissue barrier resistant to any other therapy may be treated with modified ITs. A large number of MoAbs directed against tumour cell specific antigens have been, and are being, developed. In combination with different toxins these MoAbs will result in a wide range of anti-tumour agents forming a new generation of cytostatics. In the next few years it will become more clear whether ITs are going to fulfil their potential and become a major weapon in the fight against cancer.

SUMMARY

Allogeneic bone marrow transplantation (BMT) is increasingly used as alternative treatment for patients with leukemia or lymphoma that have a poor prognosis with conventional therapy. BMT allows substantial dose escalation (up to sublethal doses) since this treatment is followed by rescue with donor bone marrow. Graft-versus-host disease (GVHD), induced by immunocompetent T cells in the graft, is a major problem in BMT and restricts this treatment to HLA-identical siblings. Therefore an alternative to allogeneic BMT is rescue infusion with autologous bone marrow aspirated prior to high dose treatment. This marrow may contain residual malignant cells the reinfusion of which will result in a relapse of the disease. In the last few years many methods for purging of bone marrow have been developed but often these are too nonspecific or have low cytotoxicity. Monoclonal antibodies (MoAbs), directed against specific differentiation antigens present on malignant T cells, and conjugated to the cytotoxic A-chain of the phytolectin ricin, appear to be highly specific cytotoxic immunotoxins (ITs) for the elimination of malignant T cells in autologous bone marrow as well as normal T cells in donor bone marrow. Ricin A-chain is an irreversible inhibitor of protein synthesis and is only effective within the cell. In this thesis parameters determining the cytotoxicity of ITs to normal and malignant T cells and the clinical use of anti-T cell ITs are discussed.

Internalization of ITs in the target cell is a prerequisite for the cytotoxic activity of the ricin A. Although one molecule penetrating into the cytosol can destroy a cell, high amounts of IT have to be internalized. Only antigens that have high internalization capacity are suitable as target for ITs. The density of such antigens on the cell surface determines the cytotoxicity of the ITs (Chapter 2). Moreover the cytotoxicity can be greatly enhanced by lysosomotropic agents like ammonium chloride depending on antigen and cell type.

Anti-T cell ITs that are well internalized (ITs directed against CD3, CD5 and CD7), separately or in a cocktail, hardly eliminated normal T cells in bone marrow whereas human T cell lines were killed very effectively depending upon antigen density. After stimulation T cells became sensitive to IT treatment suggesting that the metabolic state of the cell is an important factor. However, ITs in the presence of 20 mM

NH_4Cl killed larger number of resting T cells exceeding the elimination obtained with free MoAbs plus complement. Complete elimination of T lymphocytes in bone marrow, although overcoming GVHD, may result in a high rate of rejection and relapse after BMT. Specific depletion of T cell subpopulations therefore appear to be a more suitable approach. MoAbs directed against helper/inducer or suppressor/cytotoxic T cell subpopulations (CD4 and CD8) are hardly internalized and can only effectively used to kill cells in combination with complement (Chapters 3 and 4).

In contrast to normal T cells, ITs inhibit the protein synthesis of malignant T cells and T cell lines to a similar extent. The highly cytotoxic IT WT1-ricin A (CD7) appears to be suitable for the elimination of malignant T cells in bone marrow intended for autologous reinfusion. In most cases of acute T cell leukemia or lymphoma, malignant cells express the CD7 antigen in high amounts on their cell membrane. Besides, WT1-ricin A is continuously internalized increasing the chance of eliminating cells with a lower CD7 expression. Because no suitable culture-system exists for malignant T cells, the exact amount of cell-killing was determined by a clonogenic assay of a T cell line that expresses CD7 comparable with malignant T cells of most patients. Cell viability was reduced by more than 6-logs after incubation with the IT (Chapter 5).

Autologous BMT after WT1-ricin A treatment of the marrow of 12 patients is also described (Chapter 6). To our knowledge no other comparable numbers of transplantation of bone marrow purged by ricin A-chain IT have been published. All patients repopulated rapidly after reinfusion and the hematologic reconstitution was not impaired, though the functional reconstitution of lymphocytes was delayed. This was not caused by the method of purging and no clinical side effects were observed. Although the follow up is short and the group of patients is still small, the results of these autologous BMT are promising. Therefore, ITs may contribute to an increase in the survival and quality of life of patients undergoing treatment for cancer.

SAMENVATTING

Allogene beenmergtransplantatie (BMT) wordt in toenemende mate toegepast als alternatieve behandeling bij patienten met leukemie of maligne lymfoom, aangezien zij een slechte prognose hebben in geval van conventionele chemotherapie of bestraling. Voorafgaande aan de infusie van donorbeenmerg kunnen patienten behandeld worden met aanzienlijk hogere doses radiochemotherapie, tot zelfs sublethale doses. Graft-versus-host ziekte (omgekeerde afstotingsziekte, GVHZ), welke geinduceerd wordt door immuuncompetente T cellen in het transplantaat, is een van de belangrijkste problemen na BMT. Daarom is deze behandeling nu nog beperkt tot transplantatie van weefsel-identiek beenmerg, in de praktijk afkomstig van een nauw verwante donor, meestal een broer of zuster. De konsequentie is dat bij maximaal 15% van de patienten die in aanmerking komt voor BMT daadwerkelijk een transplantatie kan worden uitgevoerd. Een alternatief voor allogene BMT is reinfusie van autoloog beenmerg, afgenomen in remissie-fase. Dit beenmerg kan echter nog residuale maligne cellen bevatten welke na reinfusie een recidief kunnen veroorzaken. Verwijderen van deze maligne cellen is een voorwaarde wil autologe reinfusie werkelijk succesvol zijn.

In de laatste jaren zijn talrijke methoden ontwikkeld om maligne cellen uit beenmerg te verwijderen. Deze zijn echter dikwijls aspecifiek of hebben een lage effectiviteit. Monoclonale antilichamen (MoAbs), gericht tegen specifieke differentiatie antigenen op maligne T-cellen en geconjugeerd met de cytotoxische A-keten van de fytolectine ricine, genaamd immunotoxines (ITs), hebben een specifieke en bij juiste keuze een sterke cytotoxiciteit. Ricine A is een irreversibele remmer van de eiwitsynthese en is alleen effectief in het cytoplasma van de cel. Gebleken is dat ITs zeer geschikt zijn voor de eliminatie van maligne T-cellen uit autoloog beenmerg enerzijds, en normale T-cellen uit donor-beenmerg anderzijds.

In dit proefschrift is het onderzoek beschreven van parameters die de cytotoxiciteit van ITs voor maligne en normale T-cellen bepalen en de klinische ex vivo toepassing van anti-T-cel ITs. De receptor-afhankelijke endocytose van het IT is een voorwaarde voor de activiteit van ricine A. Hoewel één molekuul, na doorgedrongen te zijn in het cytoplasma, voldoende is om de targetcel te vernietigen, moet door de

hoge intracellulaire afbraak een grote hoeveelheid ITs geinternaliseerd worden. Daarom zijn alleen antigenen die een hoge internalisatie capaciteit bezitten geschikt als target. De dichtheid van deze antigenen op het celmembraan bepaalt de cytotoxiciteit van het IT (Hoofdstuk 2). Bovendien kan de cytotoxiciteit sterk verhoogd worden door lysosomotrofe agentia zoals ammonium chloride, dit is echter afhankelijk van antigeen en celtype. Ammonium chloride verhoogt o.a. de lysosomale pH waardoor de lysosomale enzymactiviteit wordt verlaagd en minder ricin A wordt afgebroken.

Anti-T-cel ITs welke in sterke mate geinternaliseerd worden (ITs gericht tegen CD3, CD5 en CD7), afzonderlijk of in een cocktail, reduceren nauwelijks het aantal normale T-cellen in beenmergaspiraten, terwijl humane T-cellijnen, afhankelijk van antigeen dichtheid, zeer effectief gedood kunnen worden. Normale rustende T-cellen worden echter gevoelig voor IT behandeling na stimulatie tot metabole activiteit met phytohaemagglutinine (PHA) suggererend dat het activatiestadium van de cel een belangrijke rol speelt. Toch kunnen ook rustende T-cellen grotendeels worden geëlimineerd door ITs indien deze worden aangeboden in aanwezigheid van 20 mM ammonium chloride. Deze eliminatie overtreft de depletie van T-cellen zoals verkregen na incubatie met MoAbs in combinatie met konijnen complement.

Totale eliminatie van normale T-lymfocyten uit donorbeenmerg voorkomt het optreden van GVHZ na BMT maar verhoogt de kans op een rejectie van het transplantaat of een recidief van de maligniteit. Een specifieke en gedoseerde depletie van T-cel subpopulaties lijkt daarom een betere benadering voor de preventie van GVHZ omdat op deze wijze cellen die een anti-leukemisch effect bezitten gespaard kunnen worden. Anti-CD7 ITs lijken hiervoor minder geschikt omdat het CD7-antigeen ook voorkomt op cellen met een anti-leukemische activiteit. MoAbs gericht tegen helper/inducer ($CD4^+$) of suppressor/cytotoxische ($CD8^+$) T-cel subpopulaties worden nauwelijks geinternaliseerd en zijn alleen effectief in combinatie met complement (Hoofdstukken 3 en 4).

In tegenstelling tot het geringe effect van ITs op normale T cellen wordt de eiwitsynthese van maligne T-cellen en T-cellijnen in sterke mate geremd. Het zeer effectieve anti-CD7 IT, WT1-ricine A, blijkt daarom wel geschikt te zijn voor de eliminatie van maligne T-cellen uit beenmerg bestemd voor autologe reinfusie. Bij de meeste patienten met

acute T-cel leukemie of lymfoom komt het CD7-antigeen in grote concentratie voor op het celmembraan van de maligne T-cel. Daarnaast wordt het CD7-WT1-ricine A-complex continu geinternaliseerd waardoor de kans op eliminatie van cellen met een lagere CD7-expressie toeneemt. Omdat tot heden geen reproduceerbare methode ontwikkeld is voor de vermeerdering van maligne T-cellen in vitro, is de eliminatie van dit celtype bepaald met behulp van een clonogene assay op een T-cellijn. De CD7-expressie op deze cellijn was vergelijkbaar met die op maligne cellen van de meeste patienten. Het aantal viabele clonogene cellen werd door incubatie met WT1-ricine A met meer dan 6-log gereduceerd (Hoofdstuk 5).

Twaalf patienten hebben een autologe reinfusie ondergaan na purgering van hun beenmerg met WT1-ricine A (Hoofdstuk 6). De hematologische reconstitutie was niet nadelig beinvloed, maar de functionele reconstitutie was vertraagd. Deze vertraging is niet veroorzaakt door de methode van celdepletie. Klinische neven-effecten zijn niet waargenomen. Uit de literatuur zijn momenteel geen waarnemingen bekend over een vergelijkbaar aantal transplantaties na beenmergpurgering met ricine A IT. Alle patienten repopuleerden snel na de reinfusie. Hoewel de periode van controle na BMT kort is en de serie patienten klein, zijn de resultaten van deze autologe BMT positief te noemen. ITs lijken daarom bij te dragen aan de verbetering van de levensperspectieven van bepaalde groepen patienten die een anti-kanker therapie moeten ondergaan.

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CURRICULUM VITAE

De auteur van dit proefschrift werd geboren op 12 december 1956 te Eindhoven. Hij bezocht de scholengemeenschap "Canisius College-Mater Dei" te Nijmegen en behaalde het diploma Gymnasium β in 1975. Daarna studeerde hij een jaar scheikunde aan de Katholieke Universiteit Nijmegen, waarna hij aangetrokken werd door de biologie. Het kandidaatsexamen biologie werd behaald in oktober 1979. Het doctoraal-examen met hoofdvak Dierfysiologie (o.l.v. Dr. J.H.F. van Abeelen en Prof. Dr. S.E. Wendelaar Bonga) en bijvakken Medische Cytologie en Histologie (o.l.v. Dr. W.M.C. Eling) en Immunologie (o.l.v. Dr. P.J.A. Capel) werd behaald in december 1982. Vanaf september 1982 verrichtte hij onderzoek in het kader van het project "Bereiding van monoclonale-antiidiotypische antistoffen gericht tegen B-CLL cellen" o.l.v. Dr. P.J.A. Capel op de afdeling Nierziekten (hoofd Prof. Dr. R.A.P. Koene) van het St. Radboudziekenhuis te Nijmegen. In september 1983 werd begonnen met het hier beschreven onderzoek op de afdelingen Bloedziekten (hoofd Prof. Dr. C. Haanen) en Nierziekten. Sedert augustus 1988 is hij een min of meer vaste medewerker van de afdeling Bloedziekten.

STELLINGEN

1. Het cytotoxisch effect van immunotoxines hangt sterker af van de mate van internalisatie van het relevante targetantigeen-immunotoxinecomplex, dan van de antigeendichtheid op het celmembraan.
(dit proefschrift)

2. Bij gebrek aan representatieve en reproduceerbare clonogene assays voor maligne cellen, bieden cellijnen een redelijk alternatief voor de bestudering van de werking van cytotoxische agentia op clonogene prolifererende cellen. (dit proefschrift)

3. Zowel normale als maligne T-cellen kunnen ex vivo effectief verwijderd worden uit beenmergaspiraten met immunotoxines welke in voldoende mate geïnternaliseerd worden. (dit proefschrift)

4. De gevoeligheid van maligne T-cellen voor immunotoxines is groter dan die van normale T-cellen. (dit proefschrift)

5. De lage gevoeligheid van normale T-cellen voor immunotoxines wordt niet veroorzaakt door een verhoogde lysosomale activiteit.
(eigen waarneming)

6. Hoewel het belang voor de in vivo functie van een groot aantal typen membraanmoleculen op T-cellen niet bekend is, bieden deze in principe wel een aangrijpingspunt om selectief normale en/of maligne T-celpopulaties met een heterogene antigeen-expressie te elimineren door deze moleculen als target voor immunotoxines te gebruiken.

7. De verhoogde kans op een recidief na een allogene beenmergtransplantatie met T-cel gedepleteerd beenmerg zou verminderd kunnen worden wanneer de eliminatie beperkt wordt tot specifieke T-cel subpopulaties, waarbij de cellen met "natural killer" activiteit in het beenmerg aanwezig blijven.

8. De neiging om risicodragende subsidieaanvragen een lager waarderingscijfer toe te kennen, werkt in de hand dat subsidies worden

toegekend aan onderzoek waarvan de uitslag al bij voorbaat vaststaat.

9. Bezuinigingen op researchgelden leiden tot vervuiling van de literatuur daar de drang tot publiceren van preliminaire resultaten verhoogd wordt.

10. De uitspraak "verder onderzoek is noodzakelijk" betekent in de praktijk een verwijzing naar een volgende publicatie.

11. Epstein-Barr virus (EBV) kan naast B-lymfocyten eveneens T-cellen infecteren en de groei dereguleren. (Kikuta et al, Nature 1988, 333, 455)

12. De "magic bullet" waar Paul Ehrlich van droomde (in: F. Himmelweit, Pergamon, New York, 1960; 1) blijkt in vivo vaak een "dum-dum kogel" effect te bewerkstelligen.

13. De mededeling door sprekers dat zij beperkt worden in hun voordracht door gebrek aan tijd kan gezien worden als promotie van eigen werk.

14. Met de introductie van automatisch openende deuren blijkt de beleefdheid om voor anderen een deur open te houden te zijn vervallen. (eigen waarneming)

15. Een computerbestand is sneller en met meer zekerheid te purgeren dan beenmerg!

Stellingen behorend bij met proefschrift:

Ex vivo elimination of normal and malignant T cells from bone marrow grafts by ricin A-chain immunotoxins

Frank Preijers,
23 februari 1989.